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UNIVERSITY OF ALBERTA

FACTORS WHICH CONTROL JEJUNAL BASOLATERAL MEMBRANE HEXOSE TRANSPORT IN RAT

BY

RAYMOND W.M. TSANG

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

DEPARTMENT OF PHYSIOLOGY

Edmonton, Alberta

Spring, 1994

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This thesis is dedicated to my parents Helen & John, my sister Sharon, and my sweetheart Keiko, all of whom never really understood what I did for two years but still loved me anyways.

ABSTRACT

The influence of luminal carbohydrates on intestinal glucose transport was studied using isolated basolateral membrane (BLM) vesicles. Luminal glucose (100 mM) perfused through the jejunum *in vivo* for 2 hrs was able to elicit an increase in the maximal transport capacity for glucose (V_{max}) 2-fold without affecting the carrier's affinity for glucose (K_m). When the perfusion duration was increased to 4 hrs, glucose (25 mM), fructose (100 mM) and sucrose (25 mM) could increase V_{max} 4-fold. Mannose (100 mM) and galactose (100 mM) were without effect on transport. The inclusion of phloridzin in the perfusate blocked the stimulatory effect seen with glucose, suggesting a requirement for the substrate to enter the cell in the signalling processes. Lactate, 3-O-methylglucose and 2-deoxyglucose were also found to stimulate glucose transport.

Cytochalasin B (CB) binding showed that there was an associated 30% increase in glucose carriers in the BLM, but only a small decrease in the number of carriers in the microsomal pool. This finding suggested that the mechanism for this rapid response may involve primarily the stimulation of activity of existing carriers along with the insertion of only a few more transporters. Western blot analysis for the GLUT2 protein revealed little change in the abundance of this glucose transporter in the BLM after luminal glucose perfusion. Northern blot hybridization for the GLUT2 gene also showed no change in the mRNA levels for this carrier under similar conditions.

Studies with fructose-inhibited D-glucose uptake in BLM vesicles indicated that other GLUT isoforms such as GLUT1, GLUT3 and GLUT4 do not play a role in this rapid response to luminal glucose. Meanwhile, diabetic animals still possess an ability to further increase BLM glucose transport along with their already elevated basal glucose absorption rates.

The intestinal hormone gastric inhibitory polypeptide (GIP) was also studied for its ability to stimulate BLM glucose transport. GIP infusion for 4 hrs at 400 pM was able to increase vesicle glucose transport 2-fold. GIP antibodies (7-5-7 and 3.65H) were able to depress the stimulation from a luminal glucose perfusion.

The role of GIP and other hormones released by GIP are discussed as possible signalling candidates for this rapid jejunal BLM response to luminal glucose.

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LIST OF ABBREVIATIONS

2-DG	2-deoxyglucose
3-OMG	3-O-methylglucose
BBM	Brush border membrane
BLM	Basolateral membrane
CB	Cytochalasin B
cDNA	Complementary deoxyribonucleic acid
FSH	Follicle stimulating hormone
GIP	Gastric inhibitory polypeptide
GLP	Glucagon-like peptide
GLUT	Facilitative glucose transporter
[I]	Inhibitor concentration
IC_{50}	Half-maximal inhibition
IDDM	Insulin-dependent diabetes mellitus
I_{max}	Maximal inhibition
IR	Immunoreactive
K_D	Rate of diffusion
K_m, K_d, B_d	Carrier-substrate affinity
LH	Luteinizing hormone
mRNA	Messenger ribonucleic acid
NIDDM	Non-insulin dependent diabetes mellitus
PBS	Phosphate buffer solution
pH	Negative log of hydrogen ion concentration

RIA	Radioimmunoassay
[S]	Substrate concentration
SDS	Sodium dodecyl sulfate
S.E.M.	Standard error of the mean
SGLT1	Na^+-dependent glucose transporter
v	Transport velocity
v/v	Volume per volume
V_{max}	Maximal transport capacity

I. INTRODUCTION

A) BACKGROUND

Glucose is the primary source of metabolic fuel which all animals can use in order to survive. The forms of glucose that can be acquired through the diet range from the single monosaccharide itself, to multi-branched glucose complexes which humans cannot digest. Because of the importance of glucose as a universal fuel for almost all of the cells in the body, the intestine must function optimally to absorb as much of this nutrient as possible when ingested. The intestine possess some plasticity in its ability to change its absorptive capacity based on the diet ingested [26]. There are also rapid changes in absorption that are short-term which occur on a meal-to-meal basis [23, 101]. If these absorptive processess do not function properly, the spillover of glucose into the large intestines can lead to osmotic and metabolic disturbances such as diarrhea and flatulence.

Glucose transport across the plasma membrane occurs through two distinct gene families of transporters. The energy dependent pathway is represented by the Na^+-dependentglucose cotransporter (SGLT1) located on the intestinal brush border membrane [147]. Utilizing an inward Na^+ gradient generated by the Na^+-K^+ATPase which is located on the basolateral membrane (BLM), the SGLT1 moves both Na^+ and glucose into the cell at a 2:1 ratio [85]. The second family of glucose transporters are facilitative (GLUT) and tend to equilibrate glucose across a plasma membrane [105, 126, 139]. Table 1.1 shows the different isoforms of glucose transporters that have been identified in various cell types.

The absorption of glucose by the small intestine occurs through a single layer of epithelial cells called enterocytes. Each cell is specialized in both its morphology and function. The enterocyte exhibits a bipolarity in its membrane structure. Facing the lumen of the intestine is the brush-border membrane (BBM), appropriately named due to its brush or comb like appearance. Here glucose can be readily transported against its concentration gradient into the cell via the SGLT1, a transport protein cloned by Wright's group [69, 114, 115, 116, 147]. The efficient activity of

Table 1.1 Facilitative glucose transporters in mammalian tissues.

Gene	Nomenclature	Major Site of Expression
GLUT1	Erythrocyte	Many tissues; high levels in human erythrocytes, brain, blood-brain barrier, placenta, kidney, colon, fetal tissues
GLUT2	Liver	Liver, pancreatic ß-cells, kidney, small intestine
GLUT3	Brain	Many tissues; high levels in brain, placenta, kidney
GLUT4	Muscle, Adipose tissue	Skeletal muscle, heart, white adipose cells, brown fat
GLUT5	Small Intestine	Small Intestine
GLUT6 (GLUT3P1)	Pseudogene	
GLUT7	Liver	Liver endoplasmic reticulum (microsomal)

this carrier ensures that as a typical meal reaches the ileum, the glucose within the lumen is nearly completely removed.

After glucose has entered the enterocyte, the movement of the hexose within the cell still remains unclear. Studies with kidney epithelium indicated transcellular flux involving the endoplasmic reticulum [8]. Waddell et al. recently discovered a novel facilitative glucose transporter which was functionally expressed only within intracellular microsomes (specifically the endoplasmic reticulum) and which shared 68% homology in its amino acid sequence to liver GLUT2 [142]. This GLUT7 protein may be the carrier which aids the rapid movement of glucose across the cell through the endoplasmic reticulum compartments. Smith's group have also shown that the distribution of amino acids within the enterocyte is not a simple relationship between molecular size and diffusion coefficient, suggesting that transcellular flux of amino acids does not occur by simple diffusion [118].

Glucose can also be metabolized once it is absorbed into the enterocyte. At luminal glucose concentrations under 10 mM, approximately 50-75% of the absorbed glucose in rats can be converted to lactate, which can then be transported out into the bloodstream [110]. However since most carbohydrate rich meals can increase luminal glucose concentrations to 50 mM, most of the absorbed glucose remains unchanged.

As glucose reaches the basolateral membrane, the facilitative carrier (GLUT2) transports the hexose into the bloodstream. Immunocytochemistry has shown that this carrier protein is present in the BLM of enterocytes populating the upper one third of the intestinal villi i.e. the cells which have matured and possess full absorptive capacity. Because GLUT2 is a high capacity, low affinity transporter, it allows for the rapid equilibration of glucose across the BLM in response to changes in intracellular glucose concentrations [139].

B) DISCOVERY OF THE BASOLATERAL POLE

The knowledge that enterocytes contain separate poles in their morphology suggested to early researchers that functional differences might also exist. In order

for absorbed glucose from the intestinal lumen to reach the bloodstream, it had to cross the hydrophobic barrier of the basolateral membrane by some mechanism other than simple diffusion. Unfortunately technical problems limited the study of the BLM due to the difficult accessibility to this 'hidden' membrane. Therefore most studies concentrated on the brush-border membrane and how sugars could be accumulated along with Na^+ [32, 84, 88, 107]. The first known study on the BLM was conducted in 1973 by Bihler et al. [9]. They devised a technique whereby isolated mouse enterocytes could be harvested and had retained both their structure and functional viability. The need for isolated cells arose from the difficulties in studying in vivo intestinal uptake due to various problems such as unstirred layers, a physical barrier residing just adjacent to the BBM [80, 144]. Bihler utilized $HgCl_2$ pretreatment in an attempt to irreversibly inhibit the Na^+-dependent glucose transport in the BBM. The remaining uptake of glucose was assumed to be across the BLM in a facilitated diffusional manner. The rationale behind the existance of a facilitative system in the BLM was based upon the necessity of the enterocyte to utilize glucose from the bloodstream for metabolism during periods in which nutrients were absent from the intestinal lumen. In addition to glucose, other substrates such as galactose, fructose and 2-deoxyglucose were also found to be possible transported substrates. Unfortunately they did not know that $HgCl_2$ possesses some ability to inhibit the BLM glucose transport system, thereby limiting their ability to further characterize the system [148].

Kimmich et al. later discovered a similar transport system in isolated chick intestinal cells [86]. They had determined that the plant glycoside phloridzin was an adequate candidate to completely inhibit BBM entry of glucose, without any effects on the facilitative carrier. They also demonstrated that phloretin, an aglucone of phloridzin, could specifically inhibit the BLM glucose flux. This discovery allowed them to directly characterize the BLM, showing that it consisted of a low affinity (high K_m) and high capacity (large V_{max}) transport system which differed from that of the Na^+-dependent BBM. This meant that the BLM possesed a transport system which could move large amounts of substrate in a relatively rapid time span, i.e. after

a carbohydrate meal. Evidence for serosal glucose transport systems were later discovered from rabbit and flounder intestine [89, 109]. All researchers agreed that the system was Na^+-independent.

The first attempt to fractionate BLM from the enterocyte was conducted by Murer et al. [108]. Based on the knowledge that the enterocyte's membrane-bound enzymes were regionally distributed, they devised a free-flow electrophoresis method to enrich BLM 5-fold. Unfortunately the final yield of their membrane preparations was rather low due to the attachment of the BLM to mitochondrial membranes during the purification procedure [121]. Several other groups along with Murer showed that isolated plasma membrane could successfully reseal and transport sugars into an osmotically active space rather than simply binding the substrates to the membranes [20, 21, 73].

Subsequently, Wright's group improved on the enrichment of BLM vesicles to 10-fold using an N_2 cavitation bomb technique and density gradient centrifugation [148]. Inhibitors such as phloretin and cytochalasin B (CB) both blocked the BLM glucose uptake, similar to the extent of inhibition found in red blood cells. The carrier's affinity for glucose (K_d = 40 mM) was found to be similar to that of the hepatocyte glucose carriers [68].

Little effort was made to further characterize the system until Maenz and Cheeseman devised a technique using a Percoll density gradient that could produce a high yield of purified BLM vesicles (18 to 20-fold) in a relatively simple and fast procedure [102]. The major advantage that their technique had over others was the tendency of their reconstituted vesicles to reseal in a predominantly outside-out orientation. This allowed them to look at the specific traits of glucose movement in both directions across the BLM. Their findings revealed that a kinetic asymmetry existed in the BLM, where the influx carrier affinity was 48 mM (similar to that of previous findings [86, 87, 148]) while the efflux affinity was 23 mM (equivalent to the movement out of the cell). This asymmetry, coupled with the action of the brush-border's Na^+-dependent transporter indicated the specialized function of the enterocyte in moving absorbed glucose towards the bloodstream. The maximal

transport capacity (V_{max}) for glucose was found to be very high, indicating either a large number of carriers existed or that transporters within the BLM possessed a high turnover rate (i.e. the ability to flip quickly from one side to another).

Glucose carrier density could be measured using a binding assay with Cytochalasin B (CB), a potent inhibitor of glucose transport in erythrocytes [76]. Since one molecule of CB can bind to one glucose transporter, investigators could estimate the number of glucose carriers within a certain population of cell membranes. CB binding data revealed that enterocytes had an affinity to CB of 0.1 μM, similar to the affinity found in red blood cells, but much lower than that of hepatocytes 1.0 μM [6], suggesting that the glucose carriers in different tissues may be different isoforms of a common glucose transporter protein.

C) ADAPTATION VERSUS REGULATION

Two distinct phenomena which can alter the movement of glucose across the intestinal BLM through different mechanisms are adaptation and regulation [23]. In adaptation, the BLM is able to change its glucose absorptive capacity in parallel to the carbohydrate composition in the diet [22, 26, 44, 45, 46, 58, 59, 60, 78, 81, 82, 133]. The mechanism involves an initial increase in glucose transport across the BLM within 3 days of switching from a low- to a high-carbohydrate diet [26]. This process occurs solely due to a proportional increase in the number of carriers. This is followed by a subsequent increase in transport at the brush-border pole after 1 week of a high-carbohydrate diet.

Regulation occurs at the BLM within a very rapid time span of 30 minutes to 6 hours, most noticeably after a carbohydrate rich meal. The mechanism involves both an increase in new glucose carriers being inserted to the BLM along with the elevation of the existing carrier's activities [29, 36, 41, 42, 105]. This thesis will focus on the phenomenon of regulation in the intestine.

D) VASCULAR CONTROL OF THE BASOLATERAL MEMBRANE

The first known experiments on the control of the intestinal BLM were

conducted by Csaky and Fischer [35]. At that time, little was known about how streptozotocin-induced diabetes in rats increased glucose transport across the intestine. Streptozotocin treatment destroys the insulin secreting cells of the pancreas, rendering the animal hyperglycemic within a few hours. In an attempt to mimic the acute phase of diabetes, rats were made hyperglycemic by infusions of glucose-saline solutions into the jugular vein. Subsequent glucose transport assays on isolated intestinal sacs revealed that there was an increase in uptake but only after 6 hrs of hyperglycemia was maintained. Furthermore, this rapid enhancement in glucose transport was blocked only by phloretin, the inhibitor of BLM glucose transport, but not by the brush-border inhibitor phloridzin. Taken together these data indicated that the presence of glucose in the vasculature could stimulate an increase in glucose transport possibly across the basolateral pole of the small intestine by the synthesis of new glucose transporter. Fischer and Lauterback were able to confirm this finding in the guinea-pig [62]. They found that short-term hyperglycemia could increase the transcellular flux of glucose, indicating that any increases in glucose transport in the BBM was matched by the BLM.

Debnam was able to localize the increase in transport after a 5 hr hyperglycemia to be primarily in the jejunum [41, 42], the region which is now known to contain the highest glucose transport activity [58, 101]. Unfortunately his technique measured the BLM indirectly through both everted sleeves [80] and in vivo uptakes (both BBM and BLM). Maenz and Cheeseman utilized the BLM vesicle protocol to study how hyperglycemic rats absorbed glucose across the BLM directly [101]. They found no significant changes to either jejunal nor ileal BBM vesicles and similarly vesicles from the ileal BLM. However, they did notice both an increase in initial rates of uptake and carrier-mediated equilibrium in jejunal BLM vesicles.

A follow-up study by this group looked at the time course of this phenomenon from 30 min to 6 hrs after hyperglycemia was induced. By comparing possible changes of CB binding to BLM vesicles to the changes in the transport capacity, they concluded that acute hyperglycemia stimulated an increase in transport activity of the existing carriers, followed later by a recruitment of a small number of transporters

into the BLM. The failure to account for the increase in transport resulting from a parallel increase in the number of carriers could be explained by a higher transport activity of the newly inserted transporters. Such carriers could differ in their kinetic traits, thus increasing transport disproportionately to carrier density.

Other substrates such as sucrose, mannose, galactose and fructose (all of which are naturally occurring sugars) could not induce this rapid response from the vasculature. Furthermore, this upregulation does not seem to involve either insulin or glucagon [25, 28], suggesting a local response involving only the intestine.

At that time, the response of the BLM to vascular glucose still remained a mystery. Why would the intestine increase its ability to absorb glucose so rapidly when there is little or no substrate present in the intestinal lumen to be absorbed? The vascular infusions which have been used previously involved a high concentration of glucose to maintain hyperglycemia. Coupled with the fact that the enterocyte possesses a facilitative glucose carrier that allows bidirectional movement of glucose, the possibility then arises that glucose in the bloodstream may 'leak' across the BLM back into the enterocyte. Whether this upregulation phenomenon requires the sensing of glucose within the enterocyte or at the intestinal lumen is still unknown, but experiments with luminally perfused glucose solutions were the next obvious step.

E) THE EFFECT OF LUMINAL GLUCOSE ON THE BLM

Only two related studies by Debnam have been done on luminal glucose perfusions to date, both of which use an indirect method of measuring BLM glucose transport. One study measured jejunal glucose uptake by two separate techniques after a 3 hr ileal glucose perfusion [79]. By measuring the transcellular flux of glucose along with the BBM flux (everted sleeves) they were able to calculate any remaining component of transport to be due to the basolateral pole. They found that luminal glucose could yield an increase in BLM transport similar to that seen after intravenous glucose. The perfusion of the ileum with glucose, followed by the increase in transport across the jejunum strongly implied that either a neural or humoral component (other than insulin or glucagon) was involved in this rapid

response [36, 79].

In summary, previous studies on the intestinal BLM used techniques which only indirectly measured transport of substrates. By measuring transcellular flux and brush-border transport, BLM transport was assumed to be the remaining component of any changes in absorptive capacity. The protocol devised by Maenz and Cheeseman to enrich functional BLM vesicles has provided an excellent tool for studying BLM transport under various conditions without the complications that intracellular metabolism had with other transport assays. Studies on hyperglycemic animals have shown that the BLM possesses an ability to rapidly change its transport capacity specifically for glucose.

F) HISTORY OF GASTRIC INHIBITORY POLYPEPTIDE

In the early 1900s, La Barre looked at gut extracts from pigs that could be used as a treatment for diabetes [96]. He coined the term 'incretin' for any factor which was released from the intestine that could stimulate an internal secretion by the pancreas. It was not until the 1960s with the advent of radio-immunoassays (RIAs) that interest in the incretins was revived. Tests showed that insulin levels rose dramatically only after an oral glucose load but not to an intravenous injection. This suggested that some factor released by the gut was affecting the pancreas [51, 103]. A few years later, Brown was able to isolate a polypeptide from the gastrointestinal tract termed gastric inhibitory polypeptide (GIP) based on its ability to inhibit gastric acid secretions from the stomach in dogs [12, 13, 16]. When GIP was infused intravenously, it enhanced serum immunoreactive insulin (IRI) and improved glucose tolerance to an i.v. glucose load [49]. However, GIP was later found to be able to stimulate insulin secretion only at a specific elevated range of blood glucose [3], leading to its alternate name of *g*lucose-dependent *i*nsulinotropic *p*olypeptide [17].

G) THE CHARACTERISTICS OF GIP

GIP is a 42 amino acid polypeptide secreted from mucosal K-cells primarily

located in the duodenum and jejunum [119, 138]. Immunocytochemical studies revealed that K-cells are most abundant in the intestinal villi and upper crypts [19]. The sequence of GIP indicates that it is a member of a family of related peptides that include glucagon, secretin and vasoactive intestinal peptide [7]. Recently, two independent groups cloned the rat GIP and found that its mRNA was distributed mainly in the duodenum and jejunum, but to a lesser extent in the ileum [71, 140]. The GIP gene was also detected by Tseng's group in the submandibular salivary gland by both RNA and RIA tests [140]. Receptors for GIP have only been detected in a hamster pancreatic insulin-secreting ß-cell line In lll [1, 2] and in muscle tissue [143] thus far.

Difficulties in measuring GIP levels arose due to the different molecular forms that are released. Gel-filtration experiments have identified three different forms. The two forms which lack any insulinotropic effect are the 8000 molecular weight species, and one which eluted in the void volume of the column [14, 15]. The predominant form which is secreted by intestinal K-cells is the 5000 MW species which does possess biological activity [92].

H) PHYSIOLOGY OF GIP

The effect that GIP has on insulin secretion has been well documented. The key to this response lies in the level of blood glucose. There is little effect of this hormone on euglycemic patients, but when blood glucose reaches a specific threshold, insulin secretion is further potentiated by GIP [3]. Diabetic patients with normally elevated blood glucose possess GIP stimulated insulin release even during a fasting state [74]. The role of GIP in both insulin-dependent diabetes mellitus (IDDM) and the non-insulin dependent diabetics (NIDDM) has been reviewed extensively by Krarup [90, 91].

Indirect evidence has also shown effects of GIP on peripheral tissues other than the pancreas. GIP was found to block both the binding of glucagon and the generation of cAMP in rat adipocytes [48]. An anti-lipolytic effect via the stimulation of lipoprotein lipase activity in cultured mouse pre-adipocytes has also been

suggested [50]. It appears that GIP increases the utilization of fatty acids to synthesize triglycerides in adipose tissue. Because adipose tissue is sensitive to the GIP actions, this hormone may play a large factor in the development of obesity.

Interactions between GIP and muscle tissue have also been suggested [77, 143]. Kahle et al. found that GIP may play a role in the muscle's increased sensitivity to insulin-induced glucose uptake when glucose homeostasis is kept normal with less insulin or in situations of stable body weight. These findings have yet to be confirmed.

GIP also has some miscellaneous effects directly on the gastrointestinal tract. These include lowering of the esophageal sphincter pressure [132], decreasing the motility of the small intestine, and a dose-dependent increase in superior mesenteric blood flow [52]. Other targets that GIP affects include the submandibular gland (alters saliva composition) and the anterior pituitary (FSH and LH secretions) [43, 111].

In conclusion, GIP should be considered as a regulatory peptide for insulin and gastric acid secretion along with a wide range of other target tissues. The important link that this hormone has to insulin appears to date to be the major function of GIP.

I) FACTORS WHICH ELICIT GIP RELEASE

The three most potent GIP secretagogues when present in the intestinal lumen are amino acids, fatty acids and glucose.

Using genetically obese hyperglycemic (ob/ob) mice, Flatt's group showed that a range of essential and nonessential neutral and basic amino acids administered orally could stimulate the release of GIP [65]. These included arginine, cysteine and histidine. Surprisingly these amino acids were also able to elicit a significant release of insulin without affecting circulating glucose levels. They suggested that GIP may play a small role in the amino acid induced release of insulin due to the different GIP and insulin response profiles. Other studies have also detected direct effects of amino acids on pancreatic insulin release [67, 120, 145].

The digestion of a typical fatty meal can yield large amounts of long-chain fatty acids. It is these hydrolyzed triglyceride byproducts that can produce the highest release of GIP (on a molar basis) when present in the lumen. Long-chain fatty acids such as oleic acid and linoleic acid can induce a 4-fold increase in the release of GIP from intestinal K-cells, while no effect is seen in the presence of either medium or short-chain triglycerides or glycerol [95, 125]. This preference for chain-length indicates that the different handling of the long-chain fatty acids along with their esterification within the smooth endoplasmic reticulum may be part of the mechanism to GIP release [30]. The release of GIP after a fat-rich meal is not insulinotropic because of the normal levels of blood glucose. This allows the animal to effectively handle the absorbed fatty acids by adipocytes without causing inappropriate hypoglycemia due to a sudden insulin release. There is also evidence that insulin (specifically the C-peptide) can exert an inhibitory effect on fat-stimulated GIP secretion [14, 34, 47, 135].

The release of GIP in response to luminal carbohydrates was examined by Sykes and colleagues in rats [136] and subsequently 9 years later by Flatt's group on ob/ob mice [64]. Both studies revealed a rapid release of GIP within 30 min of administering luminal substrates such as glucose, sucrose and maltose. Sykes was also able to show that the stimulation could be blocked by the addition of phloridzin to the luminal perfusate. They interpreted this to indicate that the substrate had to be transported into the enterocyte in order for this GIP response to occur [136]. Flatt later showed that since fructose could also stimulate the release of GIP, this response was not strictly dependent upon the activity of the Na^+-dependent glucose transporter in the BBM [64].

The negative feedback that insulin has on fat-stimulated GIP release does not seem to be present for the glucose-induced response [3, 34]. Creutzfeldt and Ebert have suggested that K-cells in the gut may possess separate receptors for fat and glucose, and that only the fat receptors are sensitive to insulin [33]. This model could then explain the large potentiation of GIP release seen from rats raised on a high-fat diet after ingesting an oral glucose meal [137].

J) GLUCAGON-LIKE PEPTIDE AS AN INCRETIN

Many gastrointestinal hormones have been isolated which posses incretin effects, specifically those structurally related to secretin. When GIP is neutralized by specific antibodies, only 50% of the luminal glucose-induced insulin secretion is abolished. This suggested that other peptides including peptide histidine methionine and peptide YY may contribute to the incretin effect [131]. The most significant of these are the glucagon-like peptides (GLP). Unlike GIP, these peptides are synthesized in both the pancreas and intestinal L-cells in the terminal ileum and colon [94, 97]. Many forms of GLP exist in the body, with the truncated GLP-1 (7-36) being the most potent in promoting insulin secretion [106]. In fact, this peptide has a greater potency in eliciting insulin release than that of GIP on a molar basis. Luminal fats and very high glucose concentrations can induce the release of GLP from the ileum, but circulating levels of this hormone do not reach as high as those of GIP under similar conditions [93, 123, 124, 146]. As nutrients rarely reach the distal ileum, the role of GLP as a true incretin has been questioned. Due to its excellent ability to suppress hyperglycemia via the insulin route, GLP remains a candidate as a potential treatment for NIDDM patients [70].

K) THE INTERACTIONS OF GIP AND GLP

Recent studies by Brubaker and Roberge have shown that a novel enteroendocrine loop may exist between GIP and GLP. One study using cultured fetal rat intestinal cells showed that GIP at a physiological dose could stimulate the release of GLP [18]. They also followed the secretion profile of both peptides after a luminal fat load and found that GIP release always increased just prior to that of GLP [124]. An explanation for the lack of response of GLP to luminal glucose may reside in a different species of GIP secreted after luminal fat versus glucose [92].

II. RESEARCH OBJECTIVE

The overall objective of this thesis is to elucidate the signals and mechanisms which control the rapid regulation of the rat intestinal basolateral membrane in response to luminal glucose. The specific aims of this project are as follows:

1) To determine which substrates within the intestinal lumen can bring about an increase in glucose transport across the BLM.

2) To elucidate the mechanism by which an increase in glucose transport can occur across the BLM after luminal glucose is presented.

3) To show if gastric inhibitory polypeptide plays a role in this rapid regulatory response.

A) RATIONALE

Aim #1:

Previous studies on the effects of hyperglycemia on the intestinal BLM transport of glucose have only linked a rapid increase in transport to acute diabetes [35]. Cheeseman and Ao later showed that this response is specific to glucose and not to other common carbohydrates [25]. The first objective then strives to show whether a more physiological signal, such as glucose within the lumen, can cause a similar increase in glucose transport across the BLM. Other carbohydrates will also be tested to determine the physiological significance of this response.

Aim #2:

The use of the protein synthesis inhibitor cycloheximide was able to block the rapid regulation after intravenous glucose, suggesting that the synthesis of new carriers was responsible for the increase in glucose transport [35]. Subsequent studies using Cytochalasin B to count the number of glucose carriers indicated that the mechanism involved more than just protein synthesis [29]. Thus, the second aim of this project was to further characterize the mechanism of this regulatory response using CB binding, Western and Northern blotting techniques.

Aim #3:

Studies on the release of GIP by luminal carbohydrates were conducted by two separate groups [64, 136]. The results from the first stated objective in this project suggested a high correlation between GIP and this rapid regulation based on the similarity of substrates which could elicit their respective responses. Therefore, the effects of GIP at various concentrations and infusion durations were tested.

III. METHODS

A) ANIMALS

Male Sprague-Dawley rats weighing 200-300 g were used for all experiments. Animals were housed three per cage and had free access to food (Purina PMI Rodent Food) and water. Sodium pentobarbitol (60 mg/kg body wt, MTC Pharmaceuticals) was used to anesthetized the animals prior to surgery via an intraperitoneal injection. Anesthesia was maintained throughout all perfusion and infusion experiments by 3-5 mg doses of pentobarbitol applied directly onto the exposed intestines. Diabetic studies were done by a single dose injection of Streptozotocin (65 mg/kg body weight, Zanosar, Upjohn) through the tail vein under methoxyflurane anesthesia (Metofane, Janssen Pharmaceutica).

B) LUMINAL PERFUSION OF THE JEJUNUM

Four animals were anesthetized simultaneously and their intestines were exposed by laparotomy. One inflow cannula was inserted 12 cm distal to the Ligament of Trietz, while an outflow cannula was inserted 35 cm distal to the first cannula. The intestinal segment was then gently flushed out with warm saline (37°C) to remove all food particles within the lumen. Care was taken not to overdistend the intestine. The intestine was replaced in the abdomen and body temperature was maintained at 37°C using heat lamps.

The inflow cannula was then connected to a Gilson Minipuls 2 peristaltic pump and a Krebs-bicarbonate solution (37°C, gassed with carboxygen) was perfused at a flow rate of 1 ml/min. The perfusing solution contained a hexose at either 25 or 100 mM concentrations. Fluid was collected from the outflow cannula and recirculated to the pump. At the end of the perfusion period of 2 or 4 hrs, the cannulas were removed and the animals were prepared for mucosal scrapings. To ensure the proper death of the animals, the hearts were cut prior to their disposal.

C) VASCULAR INFUSION OF THE JUGULAR VEIN

After four rats were anesthetized, the left jugular vein was exposed and one

silastic cannula was implanted. All vascular infusions were administered at a flow rate of 3 ml/hr containing GIP (100 - 1600 pM) or GIP antibodies (7-5-7 or 3.65 H diluted to 1:500) in an isotonic saline solution (37°C). GIP antibodies were injected slowly as an initial 1 ml bolus at the start of the infusion. At the end of the experiment, the jejunum was removed before the animals were sacrificed.

D) JEJUNAL BASOLATERAL MEMBRANE VESICLE PREPARATION

Intestinal basolateral membrane (BLM) vesicles were prepared using a previously published technique [101]. Tissues from 4 rats were pooled together to make BLM vesicles. All steps were done under ice-cold conditions to reduce degradation of the tissue. The jejunum was removed and the lumen was flushed with 15 ml of phosphate-buffered saline (pH 7.4). The mucosal surface was exposed by cutting along the anti-mesenteric border. Mucosal scrapings were then taken with a glass microscope slide with the tissue placed on top of a plastic tray, and then transferred into 60 ml of an ice-cold sucrose buffer (250 mM sucrose / 2 mM Tris-HCl (pH 7.4) / 0.1 mM Phenylmethylsulfonyl fluoride). The suspended scrapings were then homogenized with a Polytron with three 30 sec pulses. The resulting contents were equally distributed into 4 tubes and centrifuged at 2500 X g for 15 min. All centrifugation steps were done at 4°C. The fatty layer was then aspirated and the supernatant was further centrifuged at 20,500 X g for 20 min. This step yielded both a hard brown pellet made up of mostly brush-border membranes, and a fluffy white layer containing basolateral membrane. To isolate this fluffy white layer, the supernatant was slowly poured off (retained for microsomal membrane isolation) and 1 ml of sucrose buffer was added. The tube was gently swirled and the white layer was transferred to a glass-Teflon homogenizer. The membranes were sheared with 10 strokes at 1500 rpm and mixed with 4.5 ml of Percoll (12% v/v) and brought to 35 ml with sucrose buffer. Percoll is a collection of silica particles which produces a density gradient when centrifuged under specific conditions. To achieve this proper gradient, the contents were centrifuged at 48,000 X g for 1 hr. Distribution of various membranes within this gradient were pre-determined by an

enzyme assay. Ouabain-sensitive (Na^+-K^+)-ATPasewas measured since it resides primarily in the basolateral pole, while alkaline phosphatase indicated any brush-border contamination. Other enzymes associated with intracellular membranes such as the mitochondrial (cytochrome c oxidase) and endoplasmic reticulum (NADPH - cytochrome c reductase) were also measured [31, 117, 134]. Nineteen fractions from the percoll gradient were then collected and fractions 4,5 and 6 were selected to be rich in basolateral membrane vesicles. The 3 fractions of vesicles were resuspended together in a sucrose-buffer and centrifuged at 48,000 X g for 30 min, yielding a basolateral membrane pellet. This final product was diluted to 7-8 mg protein/ml in a 125 mM KSCN, 2 mM Tris HCl buffer (pH 7.4) and kept at 4°C overnight for glucose uptake measurements the following day. The amount of protein per sample was determined using a colorimetric assay (Biorad dye reagent) in a spectrophotometer (Pye Unicam PU 8600, Philips). This technique consistently yielded basolateral membrane enrichment at 18-20 fold. An independent Japanese group utilizing an identical protocol was also able to get 16-fold enrichment of BLM [105].

E) MICROSOMES

After the centrifugation steps that yielded both a white and brown pellet, the supernatant was poured off and centrifuged again at 48,000 X g for 20 min. The resulting supernatant was transferred to an ultracentrifuge and spun at 30,000 rpm for 1 hr. The pellet was then resuspended in 125 mM KSCN, 2 mM Tris HCl buffer (pH 7.4) and stored at -20°C.

F) GLUCOSE UPTAKE BY ISOLATED MEMBRANE VESICLES

All transport assays were conducted at room temperature. One 10 μl drop of vesicles was spotted onto the side of a polycarbonate tube, while another 20 μl drop of glucose uptake media was placed in close proximity onto the bottom of the tube. The uptake media contained D-glucose + D-[^{3}H]glucose (to a final vesicle protein concentration of 1.41mg/ml; D-glucose + 100 μCi D-[^{3}H]glucose/μmol D-glucose).

Uptake was initiated by a foot-switch activated vibromixer. It was determined that D-glucose uptake by BLM vesicles was linear up to 8 sec, after which uptake slowed as the vesicles filled with substrate. Therefore, 3 sec uptake durations were chosen as a reasonable approximation of initial rates to allow proper accumulation of glucose into the vesicles without the complications of possible efflux due to equilibrium exchange [101]. Uptake durations were timed by an electronic metronome. After 3 seconds, uptake was terminated by injecting 1.125 ml of ice-cold stop solution (100 mM NaCl, 1 mM Hg_2Cl_2). One ml of the resulting mixture was rapidly transferred onto a nitrocellulose filter (0.45 μm pore size, MSI) mounted in an Amicon vacuum filtration unit. The filters were washed twice with 5 ml of stop solution and dried before extraction in 5 ml of Scintiverse E scintillant (Fisher Scientific). All samples were counted on a LKB 1215 Rackbeta scintillation counter, and glucose uptake was expressed in pmol/sec/mg protein. Glucose retained within the vesicles was determined by correcting for [^{3}H]D-glucose retained by the filters in the absence of vesicles.

Because isolated vesicles show a tendency to not completely reseal themselves and they are leaky, the diffusion of D-glucose into the vesicles had to be measured. This was accomplished using a similar uptake media, with L-glucose as the replacement substrate for D-glucose.

G) FRUCTOSE INHIBITION OF GLUCOSE UPTAKE

Cross inhibition experiments were slightly modified from the earlier described assay. Varying concentrations of D-fructose were present in the D-glucose uptake media, and the uptake duration was extended to 5 sec.

H) CYTOCHALASIN B BINDING ASSAY

The binding of Cytochalasin B to glucose transporters was measured using a technique previously described [29]. An equilibrium binding technique was used due to the rapid separation (off-rate) of CB from the GLUT2 transporter. BLM vesicles were diluted to a final concentration of 1-2 mg/ml protein. Each determination of

D-glucose inhibitable [^{3}H]cytochalasin B binding was measured either in the presence of L-glucose (or sorbitol) or D-glucose. A 15-min glucose preincubation period was initiated by transferring either 1) 200 μl BLM vesicles to 500 μl L-glucose solution (1.5 M L-glucose, 3 μM cytochalasin E, and 2 mM Tris HCl, pH 7.4) or 2) 200 μl BLM vesicles to 500 μl D-glucose solution (1.5 M D-glucose, 3 μM cytochalasin E, and 2 mM Tris HCl, pH 7.4). 50 μl of a cytochalasin B solution {[^{3}H]cytochalasin B and [^{14}C]urea as required (^{3}H/^{14}C counts/min ratio = 2:1) and 2 mM Tris HCl, pH 7.4} was added to each of the L- and D-glucose mixtures after the preincubation periods. Total ^{3}H and ^{14}C disintegrations/min were measured by taking triplicate samples of 25 μl from the mixtures and placing them in scintillation vials. Three aliquots of 200 μl each were then pipetted from each mixture to six airfuge tubes and centrifuged for 30 min at 30 PSI in a Beckman airfuge (Beckman Instruments). Duplicate samples of 25 μl were transferred from each airfuge tube into scintillation tubes and the remaining supernatant was removed. The pellet was then dissolved by adding 50 μl of 1.0 M NaOH and the entire tube was dried for 1 hr in an drying oven (37°C). The pellet was subsequently neutralized with HCl and placed into a scintillation vial. ^{3}H and ^{14}C dpm were determined by liquid scintillation counting for total supernatant and pellet samples. Urea was used as a nonbinding aqueous space marker to determine the amount of unbound [^{3}H]cytochalasin B associated with the pellet.

I) WESTERN BLOTTING

Frozen scapings from the mucosa of luminally perfused or control animals were thawed and solubilized in a standard loading buffer (10% SDS, 0.5 M TRIS-HCL) containing 2-ß-mercaptoethanol. Samples were then separated by running them through a 10% SDS-polyacrylamide gel using the Minigel system at 200 V for 45 min. The proteins were then transferred onto a nitrocellulose membrane for 75 min at 4°C using 100 V and 20% methanol transfer buffer. In order to determine that the transfer was successful, Ponceau S staining was used to visualize all transferred proteins on the nitrocellulose membrane. After destaining for 5 min in

water, the membrane was blocked with 3% dried milk in PBS containing 0.05% Tween 20 (PBST) for 1 hr at room temperature. The primary anti-GLUT2 antibody was diluted to 1:1000 and incubated with the membrane overnight at 4°C. The nitrocellulose was then washed 3 times for 15 min with 3% dried milk in PBST. Protein A labelled with ^{125}I was used to tag the primary antibody, anti-GLUT2, by incubating for 1 hr at room temperature. Excess Protein A was then washed off with three 15 min washes with 3% dried milk in PBST. The nitrocellulose membrane was blotted dry and exposed to x-ray film (Kodak XAR) in a cassette and intensifying screen at -70°C for 47 hrs. The images on the developed film were scanned using an LKB Ultroscan XL laser scanner and analyzed using Gelscan XL software (Pharmacia/LKB). Molecular weights of the detected bands were determined by comparison with prestained molecular weight standards run on the same gels. Quantification of the amount of GLUT2 present was made from calculating the relative areas under the peaks obtained by the scanner and by cutting out the relevant portions of the nitrocellulose membrane and counting them in a gamma counter. Polyclonal antibodies (anti-GLUT2) were raised from rabbit against the 25 amino acid C-terminal sequence known from rat cDNA (East-Acres Biologicals, Southbridge, MA, batch # 160708B).

J) NORTHERN BLOT HYBRIDIZATION

Immediately following a luminal perfusion, jejunal scrapings were frozen and ground to a fine powder in liquid nitrogen before being homogenized in 4.0 M guanidinium thiocyanate. The homogenate was then centrifuged at 5000 g for 10 min (4°C) and the resulting supernatant was layered onto a CsCl gradient. Gradients were centrifuged at 30,000 rpm for 24 hrs at 20 °C to yield a clear RNA pellet. The DNA was aspirated, and the RNA pellet was washed with 70% ethanol at room temperature.

GLUT2 cDNA was provided by Dr.G.Bell (University of Chicago) in a pGEM4Z vector inserted between the restriction SAL 1 site. The transformants were ampicillin resistant. The plasmid was linearized with the enzyme Xba I. The

RNA riboprobe was synthesized using T7 promoter and ^{32}P-labelled CTP for 1 hr at 4°C. The probe was isolated through a sephadex G-50 column and used immediately.

Samples of total RNA (30 μg) were run along with a standard RNA ladder (GIBCO BRL) on a 1% agarose gel and then transferred to a zeta probe membrane via capillary action. The membrane was then baked at 80°C for 2 hrs in order to fix the RNA onto the membrane. Prehybridization was performed in a heat-sealable plastic bag using a standard sodium phosphate-formamide solution for 2 hrs at 50°C under constant agitation. The radioactive GLUT2 probe was allowed to hybridize with the membrane bound RNA for 18 hrs at 50°C. After hybridization, the membrane was washed throughly and then placed in a light-tight cassette and intensifying screen with x-ray film (Kodak XAR) for 48 hrs at -70°C.

K) KINETIC ANALYSIS

The total uptake of D-glucose into isolated membrane vesicles consists of two components, a carrier mediated system and simple diffusion (due to the leakiness of the resealed vesicles). Because L-glucose uptake does not involve any transport proteins, it was used as a measure for the passive entry component. The data from L-glucose uptakes were subtracted from the total D-glucose uptake to give a saturable component. This was further analyzed using Michaelis-Menten kinetics to produce constants similar to those found for enzyme kinetics. This non-linear regression analysis yields 2 constants, V_{max} (maximum saturable D-glucose uptake) and K_m (glucose-to-carrier affinity). Michaelis-Menten constants were derived from the equation [4, 5]:

$$v = \frac{V_{max} \cdot [S]}{[S] + K_m} + K_D \cdot [S]$$

where v is the absorption rate, [S] the substrate concentration and K_D is the rate of diffusion.

Fructose-inhibtion of D-glucose uptake experiments were analyzed using a similar non-saturable L-glucose component, followed by the inhibition curve constants of the IC_{50} and maximal inhibition of D-glucose uptake (I_{max}). The following equation was used for these analyses:

$$v = (I_{max} * [I]) / (IC_{50} + [I])$$

where v is uptake rate, I_{max} the maximal inhibition, and I is the inhibitor concentration.

Nonspecific [^{3}H]cytochalasin B binding was determined as binding in the presence of 1.25 M D-glucose. By subtracting total CB binding in the presence of L-glucose from that of D-glucose, we can calculate D-glucose inhibitable [^{3}H]cytochalasin B binding, that which binds only to glucose transporters. A Scatchard plot analysis was then used from four concentrations of [^{3}H]cytochalasin B to yield regression lines for bound ligand versus bound/free ligand. From this, we determined both a CB binding constant K_d, and a maximal CB binding capacity B_{max}.

All analyses were done using the computer program ENZFITTER (Biosoft, Elsevier, Cambridge, UK).

L) STATISTICS

Kinetic parameters were evaluated with unpaired Student's *t* test of the differences between two means. Statistical comparisons were considered significant at the P = 0.05 level (unless indicated otherwise). All results are expressed as means ± standard error.

IV. RESULTS

A) VESICLE TRANSPORT OF GLUCOSE

In order to determine the uptake of D-glucose by transport processes only, the leakiness of the resealed vesicles had to be taken into account. Figure 2.1 shows the uptake of D-glucose and L-glucose over a range of concentrations from isolated BLM vesicles after a 4 hr mannose (100 mM) jejunal perfusion. Total D-glucose uptake involves both carrier-mediated transport and simple diffusion. Because L-glucose uptake is non-carrier mediated, its entry into vesicles should be directly proportional to its external concentration, as illustrated by the linear uptake slope. If this diffusional coefficient, K_D, is subtracted from the total D-glucose uptake, the remaining component is the saturable carrier-mediated uptake pathway as shown in figure 2.2. With this curve, an analysis using Michaelis-Menten kinetics gave two transport variables: 1) V_{max}, or maximal transport capacity at which any further increase in substrate concentration will not change the rate of uptake (pmol/mg protein/sec) and 2) K_m, the substrate-to-carrier affinity (mM) as measured by the substrate concentration at which uptake is one-half of the V_{max}. Standard errors represent error present in the determination of the curve fit to that particular set of data. All subsequent data presented in tables represents the means of several sets of measurements and the error is that for the group of data (unless stated otherwise).

B) LUMINAL PERFUSION OF CARBOHYDRATES

Table 2.1 summarizes the result from D-glucose BLM uptake kinetics after a 2 hr jejunal perfusion. Glucose at 100 mM significantly increased the V_{max} by about 2-fold ($p = 0.05$), while mannitol, mannose and fructose produced results similar to non-infused animals. Previous studies showed that substrates had to be maintained within the lumen for at least 3 hrs in order to elicit a response [36]. Therefore, the perfusion duration was extended to 4 hrs. Table 2.2 shows the results from these perfusions under identical conditions to those in Table 2.1. Glucose at 100 mM was able to increase transport by 4-fold and only involved changes in the V_{max}, with little or no change to the K_m (Fig 2.3). Fructose, a common fruit sugar,

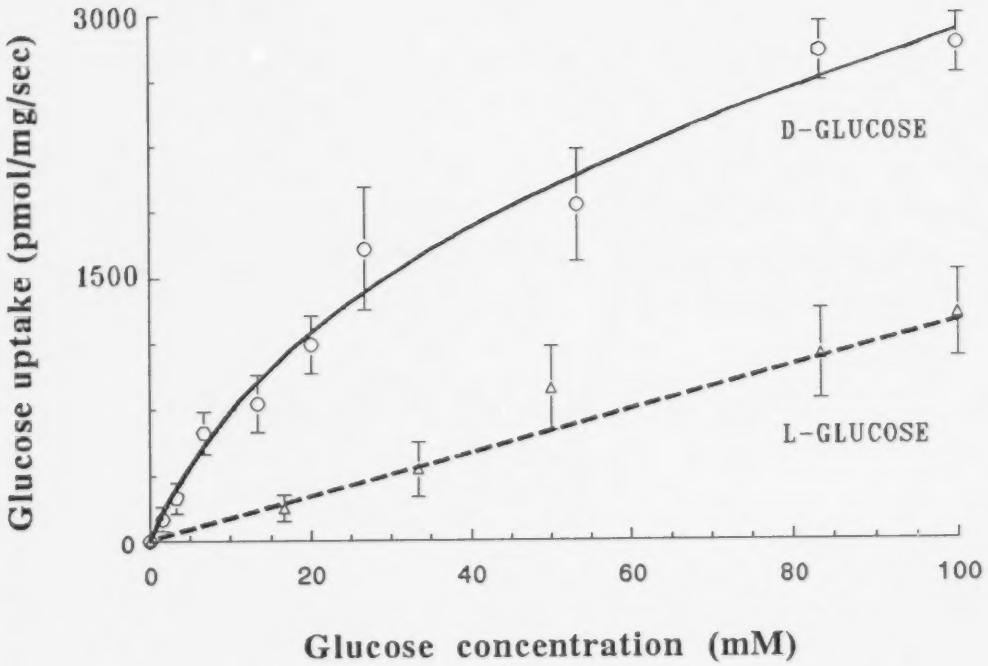

Figure 2.1 Uptake of glucose isomers by intestinal BLM vesicles prepared from the jejunum after a 4 hour mannose (100 mM) luminal perfusion. D-glucose uptake involves both carrier-mediated transport and passive diffusion (circles), while L-glucose enters via diffusion only (triangles). Data represent a single experiment using intestinal scrapings from 4 rats. Uptake of each hexose and the associated error bars were determined from triplicate samples from one batch of vesicles. (Uptake duration of 3 seconds).

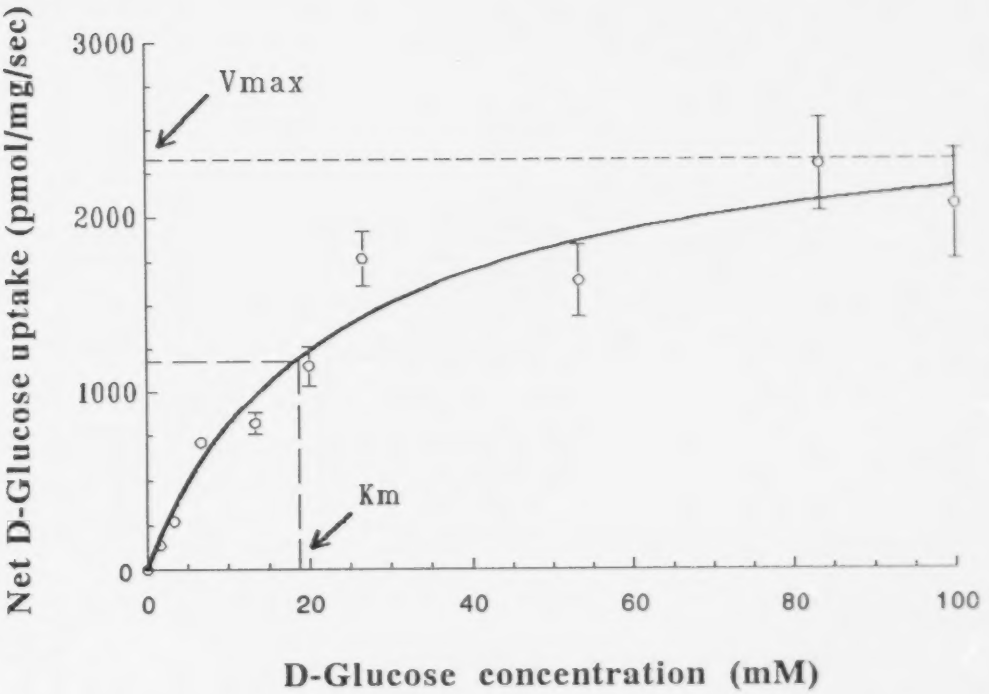

Figure 2.2 Net uptake of D-glucose from isolated jejunal BLM vesicles prepared after a luminal perfusion of 100 mM mannose for 4 hours. Data shown is taken from a single experiment, with tissues being pooled from 4 rats. V_{max} indicates the maximal glucose transport capacity. K_m is the glucose concentration at which uptake is one-half of the V_{max}. Kinetic analysis of the variables was done on the computer program Enzfitter (Elsevier) using non-linear regression analysis (see Methods). Error bars for each data point were determined from triplicate samples.

Table 2.1 Effect of 2 hour substrate perfusions on glucose uptake kinetics from jejunal BLM vesicles.

Perfused Substrate	V_{max}	K_m
Non-infused rats	912.0 ± 298.0	22.5 ± 5.8
Mannitol (100 mM)	1171.0 ± 103.0	39.8 ± 20.3
Mannose (25 mM)	795.5 ± 271.7	56.9 ± 13.7
Mannose (100 mM)	1171.0 ± 103.0	39.8 ± 20.3
Glucose (100 mM)	2389.5 ± 275.7 *	52.9 ± 3.8
Fructose (100 mM)	1037.0 ± 57.1	33.9 ± 10.2

All values were averaged from a minimum of 3 separate experiments. V_{max} values are expressed in pmol/mg protein/sec and K_m in mM. Significant differences from control (mannose perfused) animals were measured by unpaired Student's *t* tests and indicated by the * ($p=0.05$). Values are means ± S.E.M ($n=4$).

Table 2.2 Mean D-glucose uptake kinetic values from jejunal BLM vesicles of rats luminally perfused for 4 hours.

Perfused Substrate	V_{max}	K_m
Non-infused rats	912.0 ± 298.0	22.5 ± 5.8
Mannose (25 mM)	1633.9 ± 33.4	18.3 ± 1.1
Mannose (100 mM)	1673.0 ± 430.0	20.1 ± 7.9
Glucose (25 mM)	3348.7 ± 923.1 *	22.5 ± 7.6
Glucose (100 mM)	5119.7 ± 1010.7 **	39.3 ± 11.1
Fructose (100 mM)	4254.0 ± 710.3 **	39.1 ± 2.1
Sucrose (25 mM)	4409.0 ± 906.5 **	32.3 ± 13.9
Galactose (100 mM)	1926.8 ± 241.8	26.8 ± 10.1

All means were taken from at least 3 sets of experiments. V_{max} is expressed in pmol/mg protein/sec and K_m in mM. Significant differences from control animals (mannose perfused) were determined from unpaired Student's *t* tests and are indicated by the * at $p=0.05$ and ** at $p=0.01$ confidence limits. All values are means ± S.E.M ($n=4$).

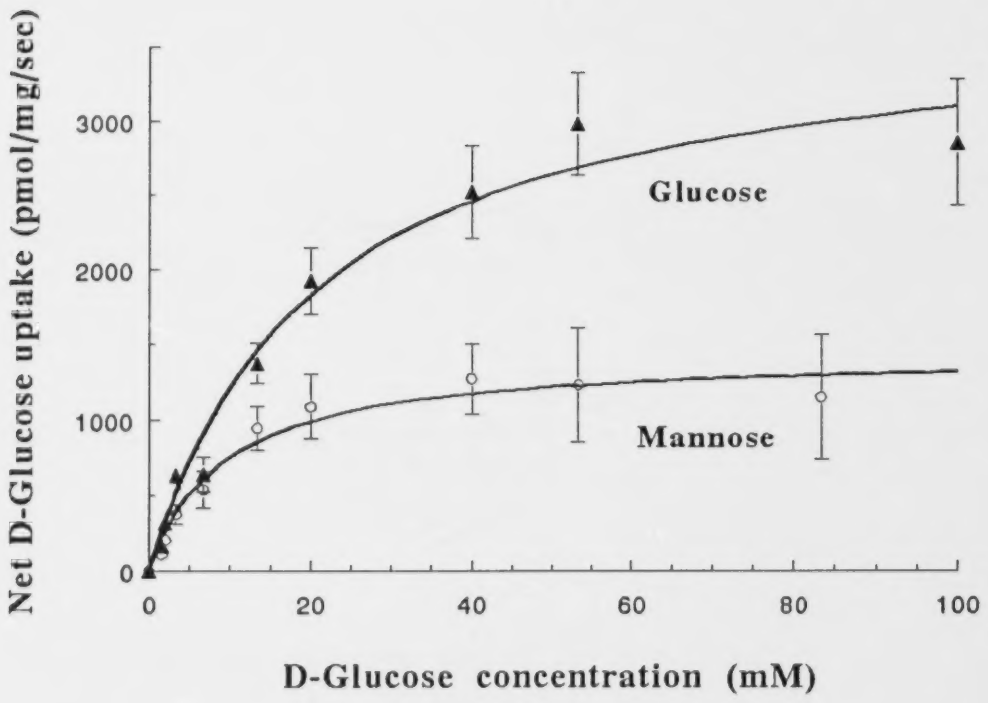

Figure 2.3 Concentration dependence curve of D-glucose uptake by BLM vesicles of rats after a 100 mM glucose (triangles) or mannose (circles) perfusion for 4 hours. Data for each hexose represents a single experiment. Uptake from mannose perfused animals did not differ significantly from non-perfused rats. Error bars were determined from triplicate samples.

also increased glucose transport by about 3-fold at 4 hrs, whereas no response was seen for the 2 hr perfusion. This suggests that this hexose requires some additional processing time in order for the stimulation to occur. All further experiments involving luminal perfusions were done for 4 hrs unless noted otherwise.

Ferraris et al. had shown that the luminal glucose concentrations in rats after a normal "physiological meal" rarely exceeds 50 mM [61]. Therefore, a more reasonable glucose concentration of 25 mM was perfused for 4 hrs. This also elicited a smaller but significant 2-fold increase in V_{max}, suggesting that this may be a normal response to physiological glucose levels in the lumen.

Sucrose, a disaccharide that consists of a glucose and fructose molecule, can be cleaved by surface hydrolases located on the BBM. The perfusion of sucrose yielded a large increase in transport even at a concentration of 25 mM. In contrast, galactose at 100 mM had no effect on glucose transport. Because this hexose is poorly metabolized by the intestinal enterocyte, this suggested that metabolism of the substrate may be a key step in the signalling process of this rapid regulation. In addition, enterocytes possess a high conversion rate of intracellular glucose to lactate [110]. Therefore, L-lactate was also perfused for 4 hrs. Only a single concentration of glucose uptake was measured from 3 separate experiments and yielded a mean uptake value of 3616 ± 852 pmol/mg protein/sec, showing a nearly 2.5-fold increase over control values ($p=0.01$). Interestingly, the perfusions of two non-metabolizable hexose analogs 3-O-methylglucose (3-OMG) and 2-deoxyglucose (2-DG) also stimulated transport. The V_{max} values from a single experiment for each substrate were 7381.1 ± 489.6 for 2-DG and 3854.5 ± 25.9 pmol/mg protein/sec for 3-OMG. All tests for significance was compared against control values determined from animals perfused with 100 mM mannose. This poorly absorbed hexose was used to maintain the osmolarity of the lumenal perfusate solution during all control experiments. V_{max} values from these rats did not differ significantly from non-infused animals or from mannitol perfused rats (Table 2.1). Luminal perfusion for either 2 or 4 hrs did not alter the positioning of the BLM vesicles on the Percoll density gradient as measured by enzyme marker assays (data not shown).

Phloridzin is a well known inhibitor of the Na^+-dependent glucose transporter located at the brush-border pole. Due to the apparent requirement for transport of the signal into the enterocyte, phloridzin was perfused along with other luminal substrates in order to block their entry into these cells. An inhibitor concentration of 2 mM was chosen as an appropriate level to overcome the unstirred layer effect [98, 99]. Table 2.3 shows that glucose at 100 mM could not stimulate glucose uptake when phloridzin was included, while fructose stimulation was only partially depressed. Meanwhile, mannose and phloridzin had no effect on glucose transport across the BLM.

C) CYTOCHALASIN B BINDING TO BLM AND MICROSOMES

Cheeseman and Maenz [29] used a CB binding assay to show the number of glucose transporters in hyperglycemic rats was disproportion to the increase in transport values. This suggested that along with an increase in the activity of existing carriers, the number of transporters also increased. The simplest explanation whereby new carriers could be inserted in such a short time would be for them to originate from an intracellular pool of membranes residing just adjacent to the BLM, i.e. the microsomal membranes. Therefore, CB binding to both the BLM and microsomal membrane fractions from luminally perfused animals was performed. Figure 2.4 shows a typical Scatchard plot of CB binding to the BLM prepared from a 100 mM glucose perfusion experiment. Non-linear regression analysis of the net D-glucose inhibitable CB binding revealed two key variables: 1) B_{max}, the maximal CB binding capacity of 31.00 ± 8.86 pmol/mg protein, and 2) K_d, the affinity of CB to the glucose transporters of 154.8 pM, as determined by the slope of the regression line. In all experiments, the K_d was found to be about 0.1 μM, similar to values in previous findings [29, 102]. In addition, the linear regression line from all perfused animals indicated that only one saturable CB-recognized binding site exists in both the BLM and microsomal membranes.

Table 2.4 shows the compiled data of B_{max} and K_d values from jejunal BLM and microsomes prepared from animals perfused with either glucose or mannose (100 mM) for 4 hrs. Glucose perfused rats showed a significant increase in the density of

Table 2.3 D-glucose uptake kinetics from rat BLM vesicles prepared after a 4 hour perfusion of 2 mM phloridzin and luminal substrates.

Perfused Substrate	V_{max}	V_{max} (with Phloridzin)
Mannose (100 mM)	1673.0 ± 430.0	1616.5 ± 215.0
Glucose (100 mM)	5119.7 ± 1010.7 **	1409.5 ± 210.8
Fructose (100 mM)	4254.0 ± 710.3 **	2705.6 ± 891.3 *

All data are averages from 3 separate experiments. Values are means ± S.E.M. Significant differences were determined by unpaired Student's *t* tests and are indicated by * at p=0.05 and ** at p=0.01. V_{max} is expressed in pmol/mg protein/sec.

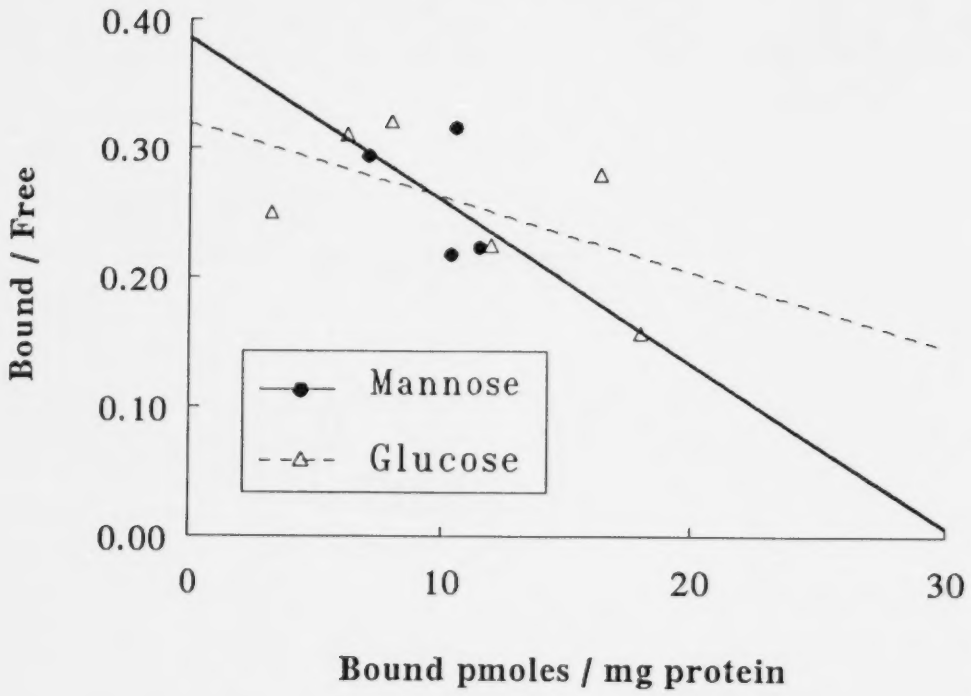

Figure 2.4 Scatchard plot for D-glucose inhibitable [^{3}H]cytochalasin B binding to BLM prepared from rats luminally perfused with 100 mM mannose (circles) or glucose (triangles) for 4 hours. Data was taken from 2 individual experiments.

Table 2.4 Kinetics of cytochalasin B binding to jejunal basolateral and microsomal membranes after a 4 hour luminal perfusion.

Membrane Fraction	Mannose (100 mM)	Glucose (100 mM)
BLM		
B_{max}	22.61 ± 5.27	31.00 ± 8.86 *
K_d	137.6 ± 42.6	154.8 ± 46.1
Microsomes		
B_{max}	21.98 ± 89.5	18.53 ± 63.4
K_d	89.5 ± 17.1	63.4 ± 22.9

B_{max} indicates the maximal CB binding capacity (pmol/mg protein/sec) and K_d is the affinity of CB to glucose transporters (pM). These constants were determined by non-linear regression analysis. All values are means from 3 experiments ± S.E.M (n=4). Significant differences were determined by unpaired Student's *t* tests and are indicated by * at p=0.05

glucose transporter sites in the BLM, as compared to control rats. If these transporters all originated from the microsomes, a parallel drop in the B_{max} should be expected from this pool. Results from these membranes revealed only a small but non-significant drop in the glucose transporter numbers.

D) IMMUNOBLOTTING FOR GLUT2 IN THE BLM

A disadvantage in using CB binding to detect changes in transporter density resides in the fact that CB cannot differentiate between different isoforms of glucose transporters. Therefore a specific measure for the GLUT2 protein was conducted using a specific polyclonal antibody raised against the rat intestinal GLUT2 transporter. There is evidence that GLUT2 is the major glucose transporter of the intestinal BLM [24]. Figure 2.5 shows the autoradiogram prepared from non-perfused rats and animals perfused for 4 hrs with 100 mM glucose or mannose. Figure 2.6 illustrates a laser densitometer scan of the autoradiogram of the bands which correspond to a molecular weight of 61 kDa. Both perfused conditions failed to increase the GLUT2 protein abundance in the BLM. Therefore, the increase in transporter density as shown by CB binding data probably involves a carrier different from the GLUT2 isoform.

E) MEASUREMENT OF GLUT2 mRNA AFTER LUMINAL PERFUSION

The possibility that this rapid regulatory response to luminal glucose involves transporter synthesis was tested by using probes specific for the mRNA of GLUT2. Figure 2.7 shows a Northern blot hybridization of total RNA under conditions of high stringency using cRNA probe raised from the pGEM4Z vector. Further analysis after a laser scan (Fig. 2.8) revealed 2 bands, one which corresponds to a 2.75 kilo base band and another at 1.77 kb. The 2.75 kilo base band corresponds to the GLUT2 mRNA as found by previous studies [27, 66]. Little change of GLUT2 mRNA was detected from rats perfused with either glucose, mannose or in non-perfused animals, suggesting that the mechanism may not involve any changes at the transcriptional level.

PICTURE REMOVED DUE TO
POOR PRINT QUALITY

Figure 2.5 Autoradiogram of a Western blot of the GLUT2 protein in BLM from animals luminally perfused for 4 hours with either 100 mM glucose (G), mannose (M) or non-perfused rats (N). Bands were measured along predetermined molecular weight markers of 69 and 46 kDa on the same gel. Four rats were used for each luminal hexose perfusion, while samples were loaded in duplicates.

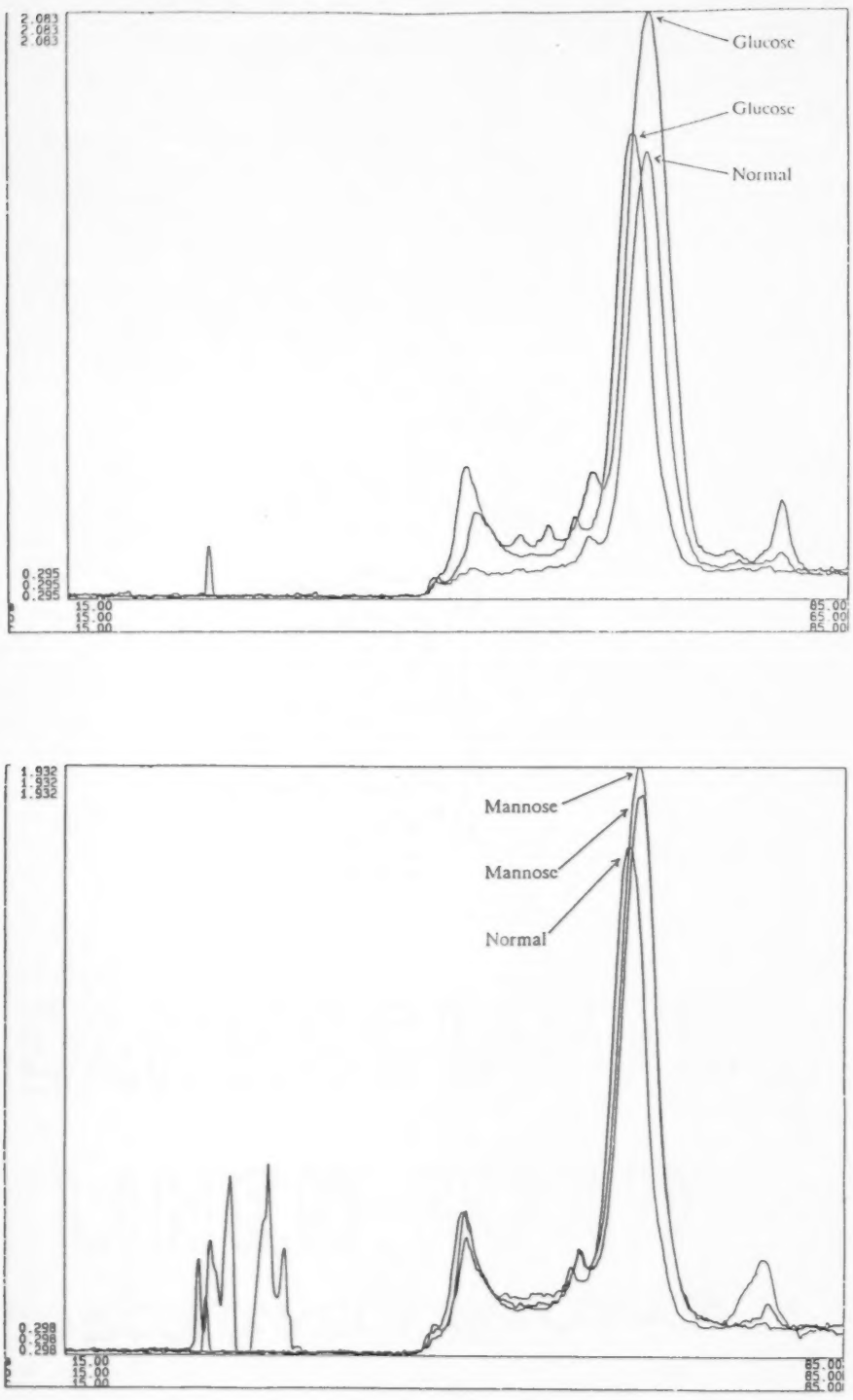

Figure 2.6 Laser densitometer scan of the autoradiogram from figure 2.5. Peaks of the scan indicated the bands to be a molecular weight of 61 kDa.

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Figure 2.7 Autoradiogram of a Northern blot hybridization for the GLUT2 gene. Animals were luminally perfused with either 100 mM glucose (G) or mannose (M) for 4 hours. Total RNA samples were taken from a single experiment per hexose perfused, and loaded in duplicates.

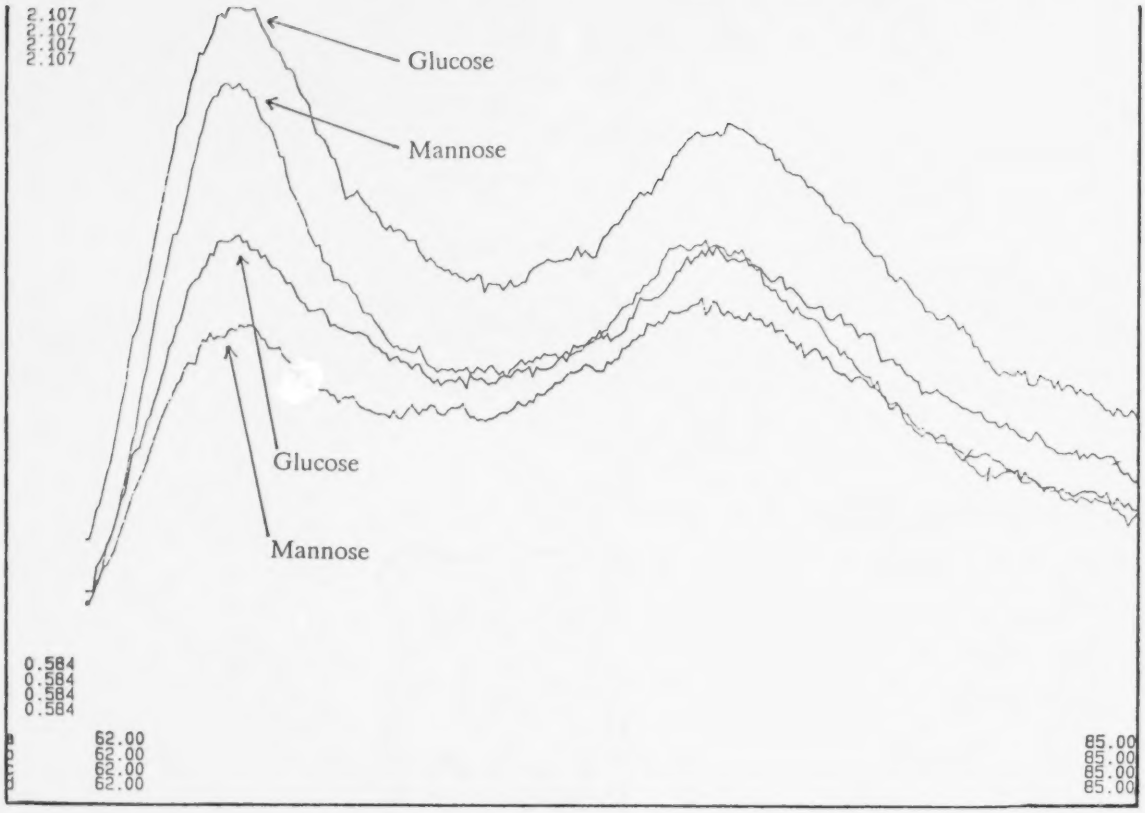

Figure 2.8 Laser densitometer scan of the Northern blot autoradiogram as seen in figure 2.7. Peaks from the scan revealed two bands, one strong peak at the 2.75 kb and a weaker signal at 1.77 kb.

F) ARE OTHER GLUT ISOFORMS INVOLVED IN RAPID REGULATION?

Previous studies have shown that GLUT1, GLUT3 and GLUT4 cannot transport fructose. However, GLUT2 has been shown to be responsible for the total flux of glucose and fructose under resting conditions. Therefore, it is possible to test GLUT isoforms other than GLUT2 by determining the proportion of D-glucose transport which can be blocked by fructose. Figure 2.9 shows the ability of fructose to inhibit glucose uptake, as determined using BLM vesicles prepared from rats luminally perfused with glucose (100 mM). The linear dotted line indicates the passive L-glucose uptake component in the vesicles (K_D). Increasing concentrations of fructose in the uptake media were capable of blocking nearly all carrier-mediated glucose uptake into BLM vesicles. The IC_{50} (inhibition constant) for fructose inhibition of glucose uptake after a glucose or mannose 4 hr perfusion ranged from 15 to 20 mM, similar to values found for the affinity of fructose to GLUT2 [24]. Results from experiments studying the glucose-inhibition of glucose uptake in BLM vesicles showed similar results to those seen using fructose as the inhibitor. All data were averaged from three separate experiments for all perfusion conditions. Taken together, these data indicate that GLUT1, GLUT3 and GLUT4 do not play a role in this rapid upregulation to luminal glucose.

G) RAPID REGULATION IN DIABETES

Diabetic rats are known to have elevated basal glucose transport rates by various mechanisms [37, 38, 39, 40, 53, 54, 55, 57]. Whether these rats still possessed a rapid regulatory response to luminal hexoses was unknown. Therefore, rats made diabetic by streptozotocin injections were perfused 4 weeks later with either glucose or mannose (100 mM) in the jejunum. Only rats with a blood glucose level above 400 mg/dl were considered to be diabetic. Table 2.5 shows the glucose uptake kinetics data from BLM vesicles prepared from these animals. The elevated basal glucose transport was illustrated by the high V_{max} values of the mannose perfused group of animals. Subsequently, glucose perfusion was able to elicit a further increase in the maximal glucose transport capacity, indicating that this regulatory component could be added to the adaptation already seen in the BLM of these

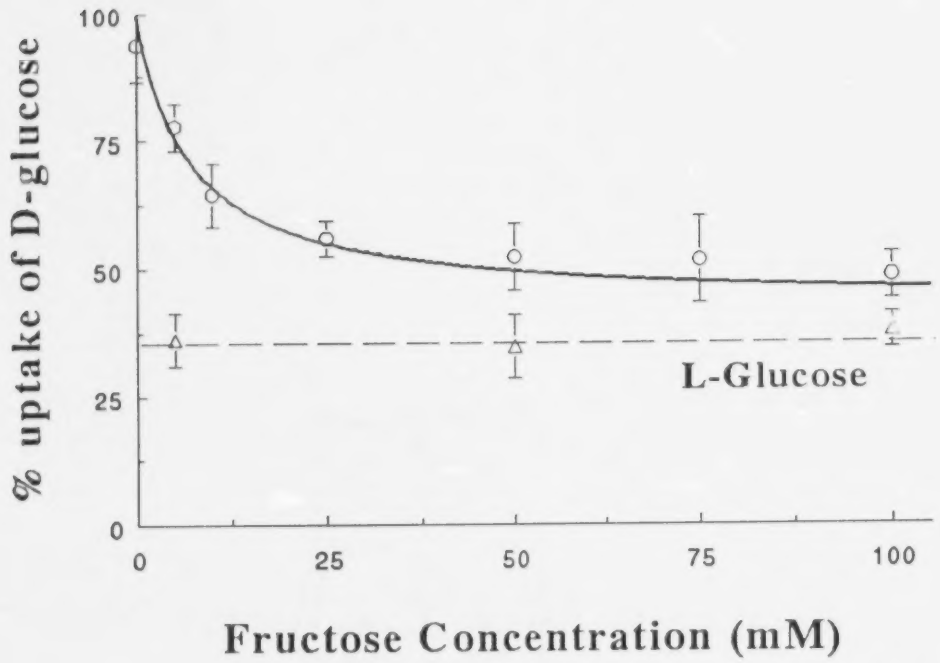

Figure 2.9 Fructose-inhibition of D-glucose uptake into BLM vesicles after a 4 hour luminal glucose perfusion. The dotted line represents uptake of L-glucose (triangles), i.e. the passive component. Total D-glucose uptake is indicated by the triangles. Uptake durations were for 5 seconds. Analysis was done using the computer program Enzfitter (Elsevier).

Table 2.5 D-glucose uptake kinetics from 4 week diabetic rats after a 4 hour perfusion of 100 mM glucose or mannose.

Perfused Substrate	V_{max}	K_m
Mannose (100 mM)	3152.7 ± 617.2	23.93 ± 13.2
Glucose (100 mM)	7737.7 ± 145.8 *	40.59 ± 5.2

All data are averaged from 3 separate experiments, ± S.E.M (n=4). Significant differences were determined by unpaired Student's *t* tests and are indicated by * (p=0.05). Vmax is expressed in pmol/mg protein/sec and K_m in mM.

diabetic rats. In all cases, increases were only seen in the V_{max}, while the K_m remained unchanged.

H) THE EFFECTS OF GIP ON BLM GLUCOSE TRANSPORT

GIP infusions for 4 hrs were done via the jugular vein and glucose transport was subsequently measured in jejunal BLM vesicles. Table 2.6 shows the results from varying concentrations of GIP in the vasculature. GIP levels after luminal glucose perfusions were shown to peak within 30 min at about 400 pM [64, 136]. Significant stimulation of glucose uptake was seen after 400 pM GIP was infused as compared to saline infused animals. It should be noted that saline infused animals had glucose uptake levels well above those of non-infused rats (approximately 4-fold higher rate of transport).

Previous experiments with luminal glucose perfusions showed that 2 hrs was adequate to cause a stimulation in BLM transport. The time which GIP was infused was also shortened to determine the minimal time required for the hormone to stimulate glucose transport. Table 2.7 summarizes the data from 400 pM GIP infused for various durations. It appears that GIP infused for 1 hr was adequate in stimulating basolateral glucose uptake.

The third set of experiments done to test the GIP hypothesis involved the use of GIP antibodies. Glucose uptake was measured in vesicles from rats injected with different forms of antibodies raised against different regions of the hormone, followed by a luminal 100 mM glucose perfusion for 4 hrs. Table 2.8 shows the results from two such antibodies. The GIP antibody 7-5-7 at a 1:500 dilution was able to inhibit about half of the stimulated glucose transport normally increased from a luminal perfusion, while the antibody 3.65H was without any significant effect.

Immunoreactive GIP levels from luminally perfused rats are shown in figure 2.10. Rats perfused with 100 mM glucose showed elevated GIP levels in the circulation within 30 min which were maintained throughout the 4 hr perfusion duration. Meanwhile, 100 mM mannose did not alter GIP levels after a 4 hr perfusion. RIA for IR-GIP was done by Dr. A. Buchan (U.B.C.)

Table 2.6 Effect of vascular GIP infusion on BLM vesicle glucose uptake.

Infused Substrate	V_{max}	K_m
Saline	4239.9 ± 379.4	21.42 ± 4.4
GIP (100 pM)	8645.0 ± 810.4 *	33.37 ± 7.2
GIP (200 pM)	6294.4 ± 989.4	32.58 ± 4.7
GIP (400 pM)	9799.9 ± 2208.8 *	36.93 ± 3.0

All values are means from at least 3 separate experiments ± S.E.M. Significant differences to control infusions was determined by unpaired Student's *t* tests and are indicated by the * ($p=0.05$). V_{max} (pmol/mg protein/sec) and K_m (mM) were determined by non-linear regression analysis.

Table 2.7 The effects of 400 pM GIP infusion durations on BLM glucose uptake by vesicles.

GIP Infusion Duration	V_{max}	K_m
Saline	4239.9 ± 379.4	21.4 ± 4.4
1 hr	7405.6 ± 1445.3 **	39.5 ± 0.4
2 hrs	8135.4 ± 774.3 **	27.4 ± 6.2
4 hrs	9158.4 ± 3990.8 *	37.8 ± 13.1

Control values were averaged from 5 experiments under 1,2 and 4 hr perfusions (not significantly different from each other). All values are from means from a minimum of 3 experiments ± S.E.M. V_{max} is expressed in pmol/mg protein/sec and K_m in mM. Significant differences were determined by unpaired Student's *t* tests and are shown by the * at $p=0.05$ and ** at $p=0.01$.

Table 2.8 Effects of GIP antibodies on D-glucose uptake from BLM vesicles after a 4 hour luminal glucose perfusion.

GIP antibody	V_{max}	K_m
Glucose only (100 mM)	3541.4 ± 391.6	29.6 ± 3.2
7-5-7	1840.3 ± 321.3 *	13.8 ± 2.4
3.65H	2181.8 ± 418.9	24.8 ± 3.8

Antibodies used were diluted to a 1:500 ratio in saline. Averaged means are from 3 experiments. V_{max} (pmol/mg protein/sec) and K_m (mM) were determined by non-linear regression analysis. Significant differences to control infusions were determined by unpaired Student's *t* tests and are indicated by the * ($p=0.05$).

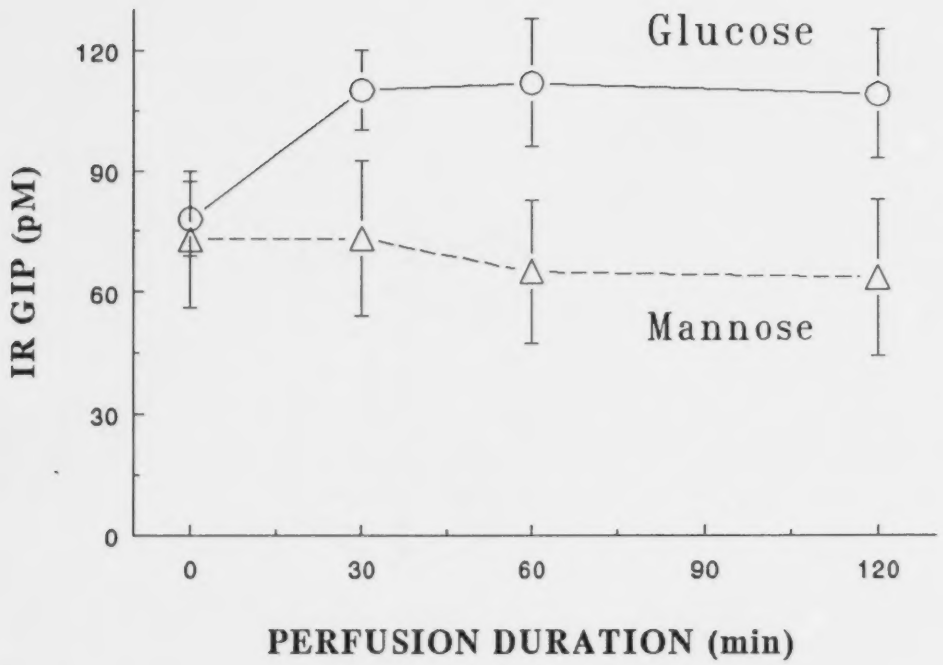

Figure 2.10 Plasma immunoreactive GIP levels during luminal perfusion of hexose. Rats were perfused with either 100 mM glucose (circles) or 100 mM mannose (triangles). Each data point was averaged from 3 separate rats ± S.E.M.

V. DISCUSSION

A) VASCULAR GLUCOSE AND INCREASED BLM GLUCOSE TRANSPORT

Csaky and Fischer were the first to identify an ability of the intestine to increase its glucose transport when hyperglycemia was induced for 6 hours [35]. Subsequent studies have shown that this increase in transport is primarily localized to the basolateral membrane (BLM) [25, 29, 36, 41, 42, 79, 101, 105]. Although the locus of the response was determined, the reason why the intestine would increase its absorptive capacity in the presence of hyperglycemia without the presence of a luminal nutrient was left unexplained. The results of this project have clearly shown that glucose in the intestinal lumen can stimulate an increase in BLM glucose transport in a similar fashion to that induced by vascular glucose. Since the exposure of either pole of the enterocyte to glucose can elevate BLM glucose transport, it appears that either the enterocyte itself is sensing glucose or that other cells could be monitoring glucose levels in the lumen and/or bloodstream. Either scenario probably involves the entrance of glucose into the cell due to the high concentration gradients generated after luminal or vascular infusions with the hexose.

The ability of the intestine to increase its absorptive capacity particularly after a carbohydrate rich meal may be a physiological response. The transit time of 4 hours would allow a typical meal to pass through the small intestines. Thus, it allows the body to extract as much nutrient from the meal as possible, to be used as metabolic fuel or stored for future needs. Also, osmotic problems can be reduced when nutrients such as glucose are not allowed to enter the large intestine. Diarrhea and flatulence are two common problems of glucose malabsorption from the small intestine.

Although the absorptive process in the intestine requires transport across two membranes, the increase in BLM transport can increase the overall transepithelial flux when a large amount of substrate is present in the intestinal lumen. Normally, transport of glucose across the BBM involves a secondary active transport process (using ATP from the Na^+-K^+ ATPase at the BLM). To prevent glucose

accumulation within the enterocyte, the exit step at the BLM must match or exceed the increased hexose entry at the BBM. Therefore, transport across the BLM must be of a very high capacity to ensure that it does not become the rate-limiting step in transepithelial flux. The argument for regulation of transport then focuses on which pole of the enterocyte can change according to specific needs. A more probable situation might involve the control of transport by both poles, with the BLM reserved for relatively rapid changes (regulation and rapid dietary changes) while longer adaptation responses (long term diet and diabetes) would involve both the brush-border and the BLM.

B) LUMINAL SIGNALS THAT CONTROL GLUCOSE TRANSPORT

The stimulation of transport by vascular infusions seems to be specific for glucose [25], but when the route of delivery was changed to the intestinal lumen, other substrates were found to elevate glucose transport. Glucose at 100 mM was able to increase transport 4-fold, with little change in the carrier affinity. At this hexose concentration within the lumen, Pappenheimer and Madara have shown that glucose and other larger molecular weight metabolites can cross the epithelium through the stimulated expansion of tight junctions between the enterocytes [100, 112, 113]. Thus, glucose can enter these cells through both the brush-border and basolateral membranes. However, a recent study by Ferraris et al. looked at the many different techniques used to measure true luminal glucose levels after a typical meal [61]. They found that previous studies which reported levels of glucose up to 200 mM were inaccurate due to poor measuring techniques. Subsequently, they devised a technique that had much improved sensitivity to glucose levels and found a concentration range between 5 to 50 mM. Therefore, a luminal perfusion of glucose at 25 mM was done to see if a more physiological signal could produce a similar result. At this lower concentration, glucose still elicited a significant stimulation of transport, suggesting that this response to luminal glucose may in fact be a physiological response.

Other carbohydrates such as fructose also increased glucose transport 2.5-fold.

Fructose is the common fruit sugar which enters the enterocyte via its own carrier, the GLUT5. Interestingly, stimulation from a fructose perfusion only occurred after 4 hours but not from 2 hour perfusion. This suggests that fructose requires a longer exposure duration to stimulate transport, possibly due to the conversion of fructose to glucose or another metabolite within the epithelium. The disaccharide sucrose also caused a large stimulation of transport even from a 25 mM perfusion. This is likely due to the fact that since surface hydrolases can cleave sucrose at the BBM prior to its entry, subsequent stimulation by the individual glucose and fructose molecules could be additive.

Hexoses which could not elicit an increase in transport included mannose and galactose. Mannose, a poorly transported and non-metabolized hexose in the intestine, yielded results similar to non-perfused and mannitol perfused animals. Galactose shares a similar transport pathway with glucose, but is only metabolized by the liver. The results from the galactose perfusion suggests that metabolism of the substrate may be a key step in the signalling process. Since up to 60% of the luminal glucose can be converted to lactate by the intestinal epithelium [110], L-lactate was luminally perfused and was found to increase glucose uptake in the rat. Two non-metabolized hexose analogs also stimulated glucose transport (3-O-methylglucose and 2-deoxyglucose). This surprising result ruled out the possibility that metabolism could be involved in the signalling process.

The apparent requirement for entry of the substrate within the epithelium as part of the signalling pathway was tested using the glucose transport inhibitor phloridzin. This plant glycoside is known to competitively inhibit the Na^+-dependent glucose transporter on the BBM, without affecting basolateral transport. A concentration of 2 mM was chosen as the minimal concentration required to overcome any unstirred layer effects generated from luminal perfusions. Phloridzin was able to abolish the stimulatory effect that luminal glucose had on BLM transport. Meanwhile, the partial inhibition of fructose-stimulated glucose transport is more difficult to explain. Phloridzin does not affect the GLUT5 transporter, but it can be converted by the intestine into its aglucone phloretin, an inhibitor which can block

the GLUT2 carrier in the BLM. Whether this side effect plays a role in reducing the response to fructose is still uncertain.

In summary, the increase in glucose transport across the jejunal BLM after luminal carbohydrate stimulation seems to be a normal physiological response. The signals which cause this stimulation include glucose, fructose and sucrose, but may ultimately be linked to the production of the metabolite lactate. Clearly, the entry of the substrate into the cell must be accomplished in order for this response to occur.

C) MECHANISMS OF INCREASING BLM GLUCOSE TRANSPORT

The increase in glucose transport after a vascular infusion was attributed to a small increase in cytochalasin B (CB) binding [29]. This was interpreted to suggest that additional transporters were inserted into the membrane; due to the lack of correlation between the level of increased transport and the number of detected carriers, the remaining activity was attributed to an increased intrinsic activity of some existing transporters. The control of functional transporters has been shown for GLUT4 from adipocytes and muscle cells, to be due primarily to the amount of phosphorylation of the carrier by kinase activating agents such as isoprenaline and phorbol esters [75, 126]. Further evidence for the control of transporter activity was shown by Cheeseman's study using the protein synthesis inhibitor cycloheximide [29]. Pretreatment with cycloheximide *in vivo* reduced the amount of glucose transport that could be stimulated by a vascular glucose perfusion, whereas glucose carrier numbers remained the same.

Results from this project have shown that there was a similar increase in CB binding to BLM prepared after a luminal glucose perfusion. However the measurement of transporter density in the microsomal membranes showed a small decrease in CB binding. This finding was somewhat in accordance to our expectations since any increase in transporter numbers in the BLM in such a short time span would probably arise from its adjacent microsomal pool. The lack of correlation between the two membrane fractions may be linked to technical

limitations. The protocol used to isolate microsomal membrane usually provides a very high yield of material, while the BLM preparation has a substantially lower yield. This could account for the inability of CB to detect a larger decrease in transporters due to the large volume of membrane that would "dilute" the signal.

An explanation for a lack of correlation between uptake measurements and CB binding data (4-fold versus 30% increase respectively) may be due to the binding characteristics of CB to glucose transporters. This compound has been shown to be able to bind to the five different isoforms of the GLUT family known, all with different binding affinities. The possibility also exists for CB to bind to quiescent carriers in the BLM, which could be activated after a luminal glucose perfusion.

The significance of controlling the activity of individual transport proteins has been discussed in a paper by Jared Diamond [44]. In this article, he proposes a hypothesis by which the intestine can change its absorptive capacity to a single meal versus long term adaptation changes. When an animal is presented with food rich in one particular nutrient, the intestine will increase its absorptive capacity for that nutrient by irreversibly increasing the number of transporters [26]. This is usually accomplished after several meals through changes in the immature cell population of the intestinal villi, in expectation that subsequent meals will also be rich in that nutrient. The basis of this response is the payoff between energy gained from the meal and energy lost in increasing its chance to absorb more of that nutrient. When this theory is applied to a single meal, the response must then be fast enough to take advantage of that passing meal, but not exceed it in duration since the intestine is uncertain if future meals will be of the same makeup. Therefore, the activation and insertion of carriers provides a suitable explanation for this rapid regulatory response without the need to synthesize a whole new population of transporters which may only be used once.

D) GLUT2 AND OTHER GLUT ISOFORM'S ROLE IN RAPID REGULATION

At the present time, the identity of the glucose transporter isoform which participates in this response has not been studied. Current data has shown that

GLUT2 is the major glucose transporter protein present in the BLM. Western blotting indicated that the abundance of GLUT2 protein remained unchanged in animals after a luminal glucose perfusion when compared to control animals. A recent preliminary report by Miyamoto et al. using a BLM vesicle isolation protocol identical to ours, showed similar immunoblotting results after a 12 hour hyperglycemia in rats fed a low-carbohydrate diet [105]. Unlike our results, they found CB binding data to parallel uptake which led them to suggest that a transporter other than GLUT2 was inserted into the BLM.

Therefore, the role of other GLUT isoforms was studied in the BLM. Fructose-inhibition of D-glucose uptake into BLM vesicles showed that nearly all of the glucose uptake could be blocked by high concentrations of fructose in the uptake medium. GLUT2 is known to be able to transport both glucose and fructose while GLUT1, GLUT3 and GLUT4 cannot transport fructose. Therefore, the ability to inhibit glucose uptake completely with fructose eliminates the possibility that these three GLUT isoforms could play a role in this regulatory response. Recent work on fructose transport across the BLM by Cheeseman has shown that GLUT2 is the sole carrier for both fructose and glucose [24]. Also, luminal perfusions of fructose for 4 hours could elevate fructose uptake in parallel to that of glucose in BLM vesicles [24]. However, a recent report from Bell's group has shown that GLUT5, the fructose carrier of the BBM, is capable of transporting glucose [122]. The stimulation of GLUT5 after a luminal glucose perfusion remains a possibility.

Another possible candidate transporter for this response was recently discovered in the endoplasmic reticulum of liver cells, appropriately termed GLUT7 [142]. This functional transporter of 52 kDa shares 68% homology in its amino acid sequence with that of the liver GLUT2. However, the C-terminal sequence in GLUT7 is different from the plasma membrane glucose transporter. Since this was the region where the antibodies used in this project were raised against, our GLUT2 antibody may not be able to recognize this GLUT7 if it was inserted into the BLM.

In summary, the mechanisms which allow a rapid increase in glucose transport seem to involve: 1) a small increase in the number of glucose transporters not related

to the GLUT2 or GLUT5 isoforms and 2) a larger increase in transport capacity that is not detectable by CB binding, suggesting that the turnover rate (i.e. intrinsic activity) of the carriers already in the membrane can be modulated by luminal or vascular glucose.

E) POSSIBLE INTERMEDIARY FACTORS IN THE SIGNALLING PROCESS

Several intestinally produced compounds have been suggested to play a role in this rapid regulatory response. Among those proposed include enteroglucagon, neurotensin or even a neural reflex [36]. Debnam's group has recently shown in a preliminary report the ability of bradykinin (10^{-7} M) to increase both the V_{max} and K_m of glucose transport across BLM vesicles after a 20 min incubation of isolated cells [128]. They also found changes to the K_m of the Na^+-dependent glucose transporter.

F) GIP AS A CANDIDATE HORMONE FOR RAPID REGULATION

The release of GIP by luminal carbohydrates has been studied by two separate groups [64, 136]. When perfused substrates which elicit GIP release are compared to those which increase BLM transport, the results strongly suggest that GIP may be the key player in linking luminal glucose to elevated BLM glucose transport. Table 3.1 shows a comparison between luminal substrates and their ability to elicit their respective responses. Both glucose and sucrose are stimulatory in all three studies, while mannose has no effect. The study by Sykes [136] was not able to detect changes in GIP release after a 30 min fructose perfusion, but Flatt's group [64] and this project both found stimulation by luminal fructose after a longer perfusion duration, indicating a requirement for additional processing of this pentose. Stimulation was also detected for the two non-metabolized hexose analogs 3-O-methylglucose and 2-deoxyglucose. The study by Sykes also showed an ability for phloridzin to block the stimulatory effect of luminal glucose on GIP release, a finding identical to that shown in this project. Therefore, the effects of vascular GIP on intestinal glucose transport were studied.

Table 3.1 Comparison of the effects of luminally perfused substrates on BLM glucose transport or GIP release.

Perfused Substrate	BLM glucose transport	GIP Secretion	
		Rat	Mice
Mannose	—	—	—
Glucose	+	+	+
Fructose	+	—	+
Sucrose	+	+	+
Galactose	—	+	+
3-O-methylglucose	+	+	+
2-deoxyglucose	+	—	+
Glucose + Phloridzin	—	—	ND

Stimulation of either BLM glucose transport or GIP secretion is indicated by the (+). No effect was detected for (−). (ND) indicates substrate effect not determined. All results for BLM glucose transport are taken from table 2.2 and table 2.3. GIP secretion data from rats were taken from Sykes et al. [150]. Luminal substrates for this study were perfused luminally for 30 min and blood was taken at the end of the perfusion duration. Data for mice GIP secretion results were from Flatt et al. [66]. Substrates listed were presented intragastrically and blood samples were taken up to 2 hours after the oral load was introduced.

G) EFFECTS OF GIP ON GLUCOSE TRANSPORT ACROSS THE BLM

Radioimmunoassays for GIP have shown that this hormone can reach levels of 400 pM in the circulation after a luminal glucose perfusion. The results after a 4 hour GIP infusion in this present study showed a 2-fold stimulation in glucose transport at an infusate concentration of 400 pM. Although this increase in transport is significant compared to control infusions, it does not parallel the 4-fold increase that was observed after a luminal glucose perfusion. This may be due to a rapid clearance rate for GIP despite a constant vascular infusion. Sheu et al. have shown that rats possess a metabolic clearance rate for GIP of about 4 ng/min [129], while Flatt's study showed that GIP levels after an oral glucose load returned to baseline within 30 minutes. Clearly an RIA to determine the concentration of GIP in blood after an intravenous infusion is required to determine what GIP levels are reached if proper comparison of these results are to be made.

The duration of GIP infusion required to stimulate glucose transport was also tested. It appears that 1 hour is sufficient to increase transport, whereas extending the infusion time to 4 hours can further elevate transport. Cheeseman and Maenz were able to detect changes in transport after a vascular glucose infusion within 30 minutes, whereas GIP levels also peak within this time for luminally perfused substrates [29, 64]. These findings strongly suggest that the increase in transport occurs rapidly in order to take full advantage of an ingested meal.

Further support for the role of GIP in rapid regulation comes from measurements of plasma IR-GIP after a luminal hexose perfusion. A 100 mM glucose perfusion was able to elicit a nearly 2-fold increase in IR-GIP levels compared to mannose perfused animals. The peak level of GIP measured in this project was lower than those reported from previous studies (about 400 pM) [64, 136]. This difference is most likely due to the perfusion of different regions of the small intestine. This project examined the perfusion of hexose only through the jejunum, while the other previously mentioned studies exposed the whole small intestine to luminal substrates, thus leading to higher GIP levels. Nonetheless, the luminal perfusion of glucose which could increase BLM glucose transport have now been shown to elevate plasma GIP levels.

The use of antibodies to block the stimulatory effect on glucose transport by GIP was only moderately successful. Three different species of GIP are known to be secreted by the intestinal K-cells, with only the 5000 MW species possessing insulinotropic activity. The antibodies used in this study were obtained from a UBC group which routinely conducts RIAs for GIP release (Dr. Alison Buchan, University of British Columbia). Unfortunately, we do not know whether the recognition site for these antibodies is the same sequence which is responsible for its stimulatory effect on glucose transport. In addition, the dilution factor chosen for these antibodies (1:500) was derived from RIA protocols and may be insufficient to completely block GIP's biological activity. Further manipulations with the GIP antibodies are required before any strong conclusions can be made at this time.

The argument for a hormone to have an effect on some tissue can be strengthened if receptors for that hormone are detectable in the target tissue. Unfortunately, GIP receptors on enterocytes have not been discovered nor have the receptors been cloned and so definitive demonstration of intestinal receptors is not yet possible. Amiranoff et al. have been able to show GIP receptor binding to a transplantable pancreatic tumor cell line but not to other tissues which show biological response to GIP, such as muscle, stomach and adipocytes [1, 2]. This pancreatic tumor cell line may have overexpressed GIP receptors, thereby allowing his "insensitive" binding assay to detect the receptors. In addition, the release of cyclic AMP was elevated by GIP in their ß-cell line, indicating a possible role for adenylate cyclase in the second messenger pathway.

There are three possible explanations for the puzzling increase in the control V_{max} transport values from the saline infused rats: 1) A change in the diet of the rats during the summer of 1992, 2) difficulties in obtaining a correct setting for a Polytron used for the initial homogenization of scraped tissue and 3) different batches of purchased radiolabelled D-glucose. A concurrent study on diabetes in our lab also found an increased transport rate for control rats (non-infused).

H) A MODEL FOR RAPID REGULATION AND GIP

Figure 3.1 shows a proposed model by which luminal glucose can elicit an

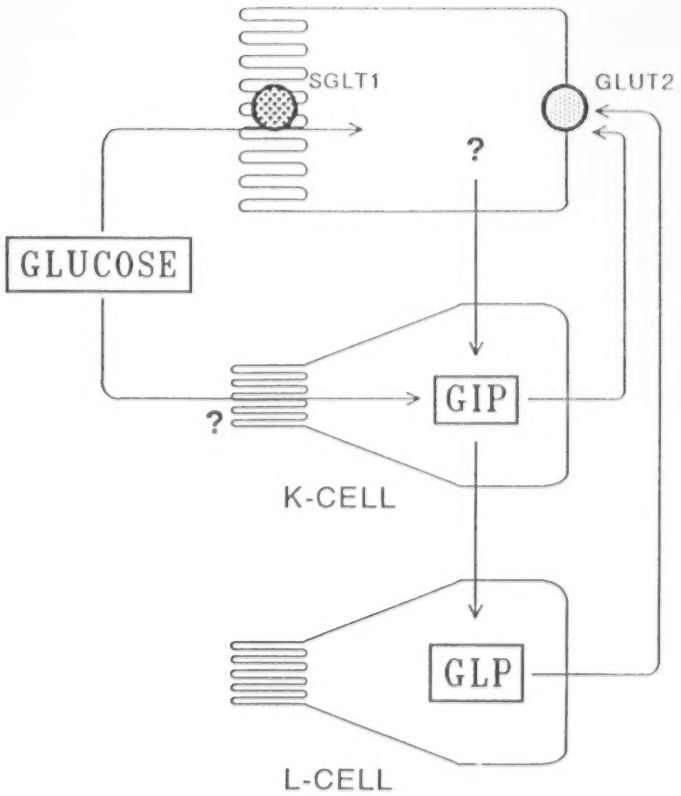

Figure 3.1 Schematic representation of events which may occur during a luminal glucose stimulation of intestinal BLM glucose transport. GIP, gastric inhibitory polypeptide. GLP, glucagon-like peptide. SGLT1, Na^+-dependent glucose transporter of the BBM. GLUT2, facilitative glucose transporter of the BLM.

increase in glucose transport across the intestinal BLM. Two pathways are suggested for glucose to stimulate GIP release. The first route may involve glucose entering the enterocyte via the Na^+-dependent transporter (SGLT1), followed by the release of an unknown substance which can trigger the secretion of GIP from the K-cells. This pathway would seem more likely due to the regional distribution of the two cell types. Since enterocytes which actively absorb nutrients are localized to the upper one-third of the villus, their ability to sense luminal glucose would be greater than that of the K-cells which are located in the crypts. Also, the unstirred layer effect would reduce the ability of luminal substrates to penetrate between the villi. However, stimulation by vascular glucose could enter the detecting cells through the BLM. Alternatively, the intestinal K-cells could sense luminal glucose directly and then release GIP. Currently, the presence of glucose transporters or receptors in the K-cells is unknown. Kieffer et al. have recently cultured porcine K-cells and shown that they release GIP in a dose-dependent manner to external glucose [83]. Further characterization of these cells will provide a better understanding of the initial pathways involved.

After GIP is released, two further routes are possible for stimulating BLM transport. The fastest route for GIP delivery would occur directly via the bloodstream to the BLM. However, a current study has shown that GIP can cause the release of glucagon-like peptides (GLP) from the intestine [124]. This hormone is synthesized by L-cells primarily located in the lower ileum and is secreted in the presence of luminal fats and glucose [18, 123, 146]. The release of this hormone after a duodenal fat infusion was shown to occur slightly after GIP secretion.

I) FUTURE STUDIES

One technique which can be used to study intestinal transport processes is the vascularly perfused intestinal preparation. This technique has been used primarily on frogs with great success due to the extended viability of their intestines [27]. By carefully controlling both perfusion media within the lumen and the intestinal vasculature, it is possible to detect very small and rapid changes in transport rates across the epithelium. In the rat, this protocol has been used to study both sugar and

nucleoside transport, but tissues only remain viable for up to 2 hours [10, 11, 63]. Nonetheless, the use of this technique to study this rapid regulatory response would prove to be a very powerful tool.

J) SUMMARY

The rapid increase in glucose transport across the intestinal BLM after a 4 hour luminal or vascular glucose perfusion appears to be a physiological response. The increased absorptive capacity would allow the animal to take advantage of a single meal that has been ingested. Stimulation of transport appears to require glucose and other substrates to enter either the enterocyte or other cells which can detect the substrate. The mechanism of this process appears to involve a rapid increase in the activity of existing carriers in the membrane, followed by insertion of additional carriers for glucose. Both GLUT2 and GLUT5 have been ruled out as the inserted carrier in question, although other transporters from the GLUT family remain as possibilities. Finally, preliminary results suggest that GIP may be the link in this substrate regulated transport process within the small intestine.

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